

# DRUG TRANSPORTERS AND NHERF PDZ PROTEINS

by

Dustin R. Walsh

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This thesis was presented

by

Dustin R. Walsh

It was defended on

October 6, 2015

and approved by

Thomas D. Nolin, Pharm.D., Ph.D., Assistant Professor, Pharmacy and Therapeutics

Peter A. Friedman, Ph.D., Professor and Vice Chair, Academics, Pharmacology and Chemical  
Biology

Samuel M. Poloyac Pharm.D., Ph.D., Associate Dean for Graduate and Post Doctoral  
Programs, Professor

Philip E. Empey Pharm.D., Ph.D., Assistant Professor, Pharmacy and Therapeutics

Thesis Advisor: Thomas D. Nolin, Pharm.D., Ph.D., Assistant Professor, Pharmacy and  
Therapeutics

Thesis Co-Advisor: Peter A. Friedman, Ph.D., Professor and Vice Chair, Academics,  
Pharmacology and Chemical Biology

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Dustin R. Walsh, Pharm.D., M.S.

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Drug transporters govern the absorption, distribution, and elimination of pharmacologically active compounds. Members of the solute carrier (SLC) and ATP binding-cassette (ABC) drug transporter family mediate cellular drug uptake and efflux processes, thereby coordinating the vectorial movement of drugs across epithelial barriers. To exert their physiological and pharmacological function in polarized epithelia, drug transporters must be targeted and stabilized to appropriate regions of the cell membrane (*i.e.*, apical versus basolateral). Despite the critical importance of drug transporter membrane targeting, the mechanisms that underlie these processes are largely unknown. Several clinically significant drug transporters possess a recognition sequence that binds to **PSD-95/Drosophila discs large/ZO-1** (PDZ) proteins. PDZ proteins, such as the Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor (NHERF) family, act to stabilize and organize membrane targeting of multiple transmembrane proteins including many clinically relevant drug transporters. These PDZ proteins are normally abundant at apical membranes, where they tether membrane-delimited transporters. NHERF expression is particularly high at the apical membrane in polarized tissue such as intestinal, hepatic, and renal epithelia, tissues important to drug disposition. Several recent studies have highlighted NHERF proteins as determinants of drug transporter function secondary to their role in controlling membrane abundance and localization. Mounting evidence strongly suggests that NHERF proteins may have clinically significant roles in pharmacokinetics and pharmacodynamics of several pharmacologically active compounds and may affect drug action in cancer and chronic kidney disease. For these reasons, NHERF proteins represent a novel class of post-translational mediators of drug transport and novel targets for new drug development.

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## PREFACE

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## I. INTRODUCTION

Plasma membrane transport proteins play a central role in mediating the disposition or pharmacokinetics, *i.e.*, absorption, distribution, and elimination of a broad spectrum of endogenous and exogenous compounds including pharmacologically active compounds and clinically used medications (Nigam, 2015). Such transporters are now acknowledged also as clinically significant determinants of drug response, *i.e.*, pharmacodynamics (Konig et al., 2013). Although broadly expressed, drug transporters exhibit highest abundance in intestine, liver, and kidney; tissues important for drug disposition (Giacomini et al., 2010). Furthermore, drug transporters expressed at the blood-brain barrier and the placenta serve an additional protective role to minimize exposure of the brain and fetus, respectively, to toxic compounds.

Solute carrier (SLC) and the ATP-binding cassette (ABC) transporters form the two major superfamilies of drug transporters. SLC transporters primarily mediate cellular uptake of drugs by facilitated diffusion or ion-coupled secondary active transport. ABC transporters function as primary energy-dependent active transporters and rely on the hydrolysis of ATP to energize cellular drug efflux. Functionally, SLC and ABC drug transporters work in concert to couple the vectorial movement of drugs and drug metabolites across epithelial barriers. The clinical impact of drug transporters in pharmacotherapy highlights the need to fully characterize their regulatory mechanisms (Hillgren et al., 2013).

Epithelial cells that form the intestinal, hepatic, and renal barriers display characteristic apical-basolateral membrane polarity. Drug transporters that are expressed in these cells must be targeted and stabilized at the appropriate membrane domain (*i.e.*, apical or basolateral) to elicit their physiologic and pharmacologic function (Kato, 2007). A key role of drug transporters

located at epithelial barriers is to limit systemic drug exposure. Oral drug absorption is accomplished by an array of drug transporters that are distributed throughout the intestinal epithelium. In the liver, transporters coordinate the hepatobiliary disposition of drugs and serve a principal role in biliary drug excretion. Together, intestinal and hepatic drug transporters play a major role in mediating drug bioavailability. Drug transporters expressed in renal proximal tubules move organic solutes across the proximal tubule epithelium. Drug transporters in the kidney are significant contributors to renal drug elimination and are involved in both secretion and reabsorption into and from the tubular lumen. At all of these epithelial barriers, drug transporters are localized to specific membrane domains, which is critical to their functional role in drug bioavailability, disposition, and response. Defining the mechanisms that determine drug transporter abundance and membrane-delimited localization is an emerging line of research.

PDZ proteins are named for the common structural domain shared with the postsynaptic density protein (PSD), *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (ZO-1). In addition to their involvement in the assembly of multi-protein signaling complexes, PDZ domain-containing proteins also regulate membrane abundance and the asymmetrical sorting of proteins to specific membrane domains in polarized epithelia (Padanyi et al., 2010; Shenolikar et al., 2002; Shenolikar and Weinman, 2001). PDZ proteins recognize and bind to target proteins by specific internal and carboxy-terminal amino acid sequences, referred to as PDZ ligands (Songyang et al., 1997; Stricker et al., 1997). PDZ domains are classified based on the structural binding motif of the PDZ ligand with which they interact (Table 1). Class I PDZ proteins interact with ligands terminating in the sequence -X-[S/T]-X- $\phi$ , where X is promiscuous and  $\phi$  is a hydrophobic residue, generally Leu, Ile, Val, or Met. Class II PDZ proteins prefer ligands terminating in -X- $\phi$ -X- $\phi$  and Class III prefer a PDZ binding motif of the form -[D/E]-[K/R]-X- $\phi$ . However, PDZ proteins may also recognize internal peptide fragments from target proteins. Residues up to 18 amino acids upstream from the carboxy-terminus can participate in target recognition (Mahon and Segre, 2004). These non-canonical

TABLE 1

## PDZ ligand recognition classification

Class	C-terminal consensus sequence of PDZ ligand <sup>‡</sup>
I	-X-[S/T]-X-φ
II	-X- φ -X-φ
III	-[D/E]-[K/R]-X-φ

<sup>‡</sup> X is permissive indicating any amino acid; S/T are serine and threonine, φ hydrophobic residue, typically L, I, V. D/E and K/R are aspartate/glutamate, and lysine/arginine, respectively.

binding modes may also be important for establishing target specificities. Several clinically significant SLC and ABC drug transporters harbor a carboxy-terminal PDZ ligand and therefore may bind PDZ proteins. Mounting evidence highlights the impact of PDZ proteins in regulating the membrane abundance, localization, and function of a growing number of drug transporters (Karvar et al., 2014; Park et al., 2014; Zheng et al., 2014).

The Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factors (NHERFs) are a family of PDZ proteins that also act as mediators of drug transporter function secondary to their role in organizing drug transporter membrane abundance and localization. NHERF proteins alter drug transporter function *in vitro*, as well as drug absorption and disposition *in vivo* (Park et al., 2014). NHERF proteins are highly expressed at apical membranes of polarized epithelial cells of the kidney, intestine, and liver, where they interact with numerous drug transporters from both SLC and ABC superfamilies (Kato et al., 2006; Kato et al., 2004; Sugiura et al., 2011). The majority of reported interactions are mediated through canonical class I PDZ ligands, though interactions with drug transporters possessing class II and III PDZ ligands also have been described (Kato et al., 2004; Shimizu et al., 2011; Sugiura et al., 2008). Hence, NHERF proteins may have an indirect effect on drug response attributable to their influence on drug absorption and disposition in these important pharmacological barriers. Over the last decade, PDZ proteins have gained considerable attention as post-translational regulators of drug transporter function (Choi et al., 2011; Kato et al., 2006; Miyazaki et al., 2005; Noshiro et al., 2006; Park et al., 2014; Sugiura et al., 2006; Sugiura et al., 2008; Sugiura et al., 2010; Sugiura et al., 2011; Wang et al., 2014; Zheng et al., 2014). To highlight these new findings, we review the current knowledge of drug transporter regulation by PDZ proteins and address the association of PDZ proteins with drug disposition and response in select disease states.

## II. DRUG TRANSPORTER STRUCTURE, FUNCTION, AND TISSUE LOCATION

### A. Solute Carrier (SLC) Drug Transporters.

SLC drug transporters are classified into the *SLC22A* and *SLCO* gene families.<sup>1</sup> The *SLC22A* genes include the organic anion transporters (OATs), organic cation transporters (OCTs), and organic carnitine transporters (OCTNs). The *SLCO* gene family consists of the organic anion-transporting polypeptides (OATPs). Members of both gene families are primarily involved in cellular drug uptake and are abundantly expressed at the epithelium in the liver, intestine, and kidney (Figure. 1) (Giacomini et al., 2010). Although most SLC superfamily drug transporters display overlapping substrate specificities, OATs, OCTs, and OCTNs transport anions, cations, and zwitterions, respectively, whereas OATPs transport larger hydrophobic anions.

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<sup>1</sup> Uppercase denotes human genes or gene products and lowercase denotes rodent genes or gene products

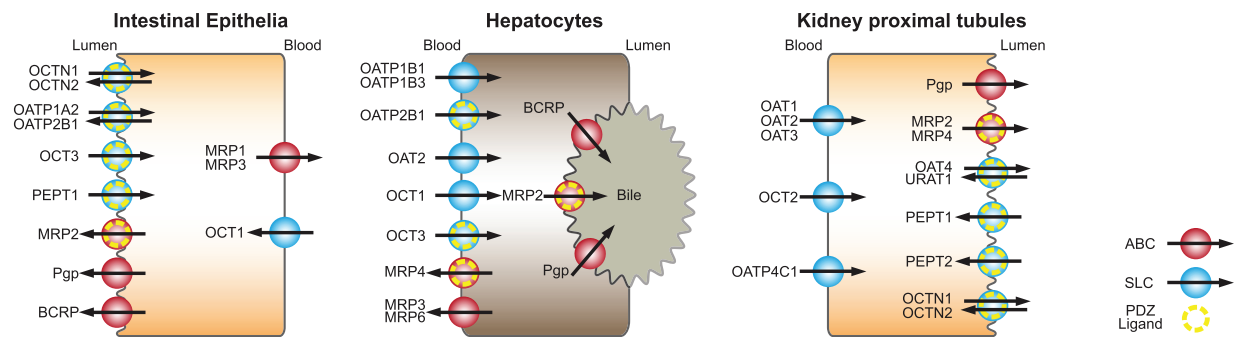


Figure 1

## 1. SLC22A Family.

Transporter proteins encoded by the SLC22A gene family include, OCT1-3 (SLC22A1-3), OCTN1-2 (SLC22A4-5), OAT1-3 (SLC22A6-8), OAT4 (SLC22A11), and URAT1 (SLC22A12).

The prototype membrane topology of the SLC22A family comprises 12  $\alpha$ -helical transmembrane domains (TMD), an intracellular N-terminus, a large extracellular loop between TMDs 1 and 2, which harbors several putative N-glycosylation sites, a large intracellular loop between TMDs 6 and 7 that contains additional glycosylation and phosphorylation sites, and an intracellular C-terminus (Figure. 2). Tissue expression and membrane localization of selected SLC22A drug transporters is summarized in Figure 1. SLC22A drug transporters and selected substrates are summarized in Table 2.

### a. Organic Cation Transporters (OCTs).

OCT1 (SLC22A1), OCT2 (SLC22A2), and OCT3 (SLC22A3) mediate transport of organic cationic drugs such as metformin, histamine H2 receptor antagonists, and platinum-based drugs. OCT1-3 share three common functional characteristics. First, substrate transport is bidirectional, that is, OCTs mediate both uptake and efflux processes. Second, transport occurs by passive facilitated diffusion that is driven by the negatively charged intracellular membrane voltage. The rate of substrate uptake and efflux therefore depends upon the electrochemical gradient and is sensitive to changes in the membrane potential. Third, OCT-mediated transport



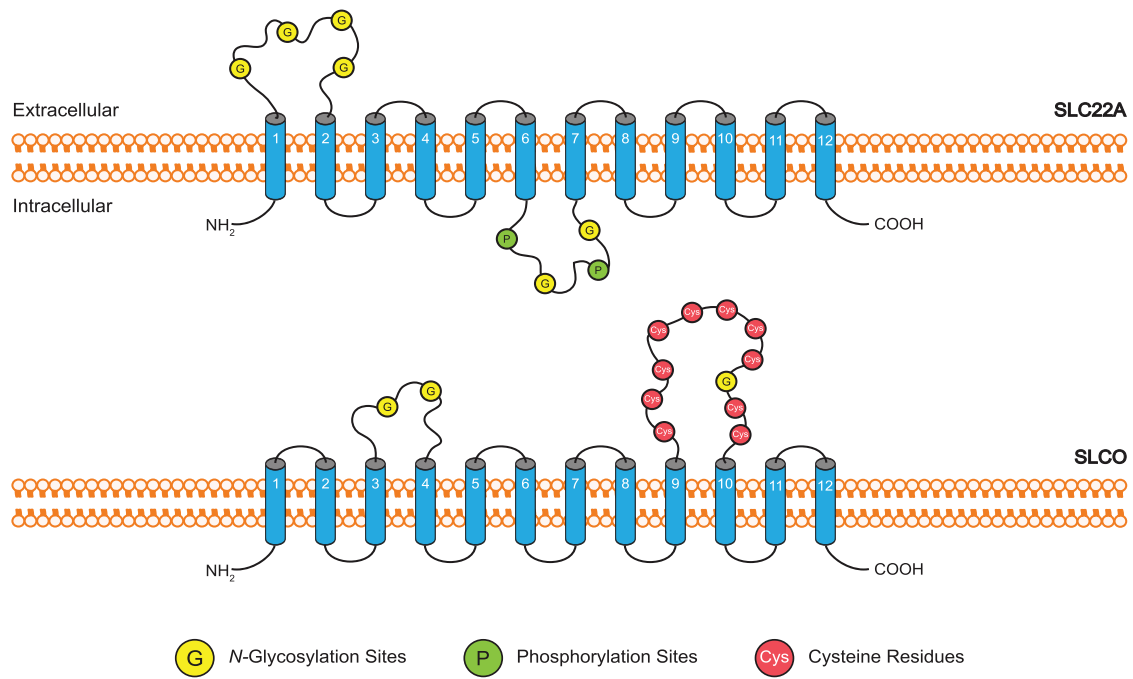


Figure 2

TABLE 2  
Selected Drug Transporters

Transporter	Superfamily	Family	Gene Name	PDZ Ligand	Selected Substrates
OCT1	SLC	SLC22	<i>SLC22A1</i>	---	Metformin, oxaliplatin
OCT2	SLC	SLC22	<i>SLC22A2</i>	---	Metformin, ranitidine, varinicine
OCT3	SLC	SLC22	<i>SLC22A3</i>	R-S-H-L	Histamine, epinephrine
OCTN1	SLC	SLC22	<i>SLC22A4</i>	L-T-A-F	Quinidine, verapamil, ipratropium, ergothioneine
OCTN2	SLC	SLC22	<i>SLC22A5</i>	S-T-A-F	Carnitine, amisulpride, cephaloridine
OAT1	SLC	SLC22	<i>SLC22A6</i>	---	Lamivudine, acyclovir, ciprofloxacin
OAT2	SLC	SLC22	<i>SLC22A7</i>	---	Bumetanide, erythromycin, paclitaxel
OAT3	SLC	SLC22	<i>SLC22A8</i>	---	Furosemide,

					bumetanide
OAT4	SLC	SLC22	<i>SLC22A11</i>	S-T-S-L	Bumetanide, urate, methotrexate
URAT1	SLC	SLC22	<i>SLC22A12</i>	S-T-Q-F	Urate
OATP1A2	SLC	SLCO	<i>SLCO1A2</i>	K-T-K-L	Statins, fexofenadine
OATP1B1	SLC	SLCO	<i>SLCO1B1</i>	---	Statins, repaglinide, olmesartan
OATP1B3	SLC	SLCO	<i>SLCO1B3</i>	---	Statins, telmisartan, fexofenadine
OATP1C1	SLC	SLCO	<i>SLCO1C1</i>	E-T-Q-L	Triiodothyronine (T <sub>3</sub> ), Thyroxine (T <sub>4</sub> )
OATP2B1	SLC	SLCO	<i>SLCO2B1</i>	D-S-R-V	Statins, fexofenadine
OATP3A1	SLC	SLCO	<i>SLCO3A1</i>	E-S-V-L	Benzylpenicillin, vasopressin
OATP4A1	SLC	SLCO	<i>SLCO4A1</i>	Q-S-S-V	Estradiol-17 $\beta$ - glucuronide
OATP4C1	SLC	SLCO	<i>SLCO4C1</i>	---	Digoxin, methotrexate, sitagliptin

PEPT1	SLC	SLC15	<i>SLC15A1</i>	Q-K-Q-M	Cephalexin, valacyclovir, enalapril
PEPT2	SLC	SLC15	<i>SLC15A2</i>	K-T-K-L	Cephalexin, valacyclovir, enalapril
MDR1 (Pgp)	ABC	ABCB	<i>ABCB1</i>	---	Digoxin, loperamide
BCRP	ABC	ABCG	<i>ABCG2</i>	---	Statins, methotrexate
MRP1	ABC	ABCC	<i>ABCC1</i>	---	Citalopram, methotrexate, Leukotriene C4
MRP2	ABC	ABCC	<i>ABCC2</i>	S-T-K-F	Olmesartan, methotrexate, etoposide
MRP3	ABC	ABCC	<i>ABCC3</i>	---	Estradiol-17 $\beta$ - glucuronide
MRP4	ABC	ABCC	<i>ABCC4</i>	E-T-A-L	Adefovir, topotecan, methotrexate
MRP7	ABC	ABCC	<i>ABCC10</i>	D-T-R-L	Docetaxel, paclitaxel, viscristine, vinblastine

is independent of sodium and pH. OCTs are expressed in the kidney, intestine, and liver, where they function primarily as basolateral uptake transporters. However, OCT3 may be expressed at both basolateral and apical membranes (Muller et al., 2005; Nies et al., 2009). For example, OCT3 is localized to basolateral/sinusoidal membranes of hepatocytes, and apical membranes of enterocytes. OCT3 is expressed in renal proximal and distal tubules but additional studies are needed to define OCT3 membrane localization in the kidney (Wu et al., 2000b).

OCTs exhibit broad selectivity. Common substrates include hydrophilic, low molecular weight organic cations (Table 2). OCT1 and OCT2 are drug transporters of emerging clinical significance due to their potential influence on drug disposition and response. For example, OCT1 is an important factor in the pharmacotherapy of diabetes and chronic myeloid leukemia (CML) (Grinfeld et al., 2013; Koren-Michowitz et al., 2014; Mahrooz et al., 2014). OCT1 is thought to be liver-specific; it is predominately expressed on basolateral/sinusoidal membranes of hepatocytes and mediates drug uptake (Gorboulev et al., 1997; Zhang et al., 1997). Although few endogenous OCT1 substrates have been identified, evidence suggests that hepatic OCT1 may mediate the uptake of biogenic amines such as serotonin (Boxberger et al., 2014).

Moreover, commonly prescribed medications such as diphenhydramine, fluoxetine, imatinib, and verapamil, possibly inhibit OCT1-dependent serotonin uptake, a finding highlighting the possibility that drugs may interfere with transport and elimination of endogenous substrates at the transporter level. OCT2 is found exclusively in the kidney at basolateral membranes of proximal tubule cells, where it facilitates secretion of drugs such as cisplatin and oxaliplatin (Gorboulev et al., 1997; Iwata et al., 2012; Urakami et al., 2002). OCT3, in contrast to OCT1 and OCT2, displays a much broader tissue expression pattern. In addition to expression in the intestine, liver, and kidney, OCT3 mRNA levels can be detected in the placenta, heart, brain, and skeletal muscle (Hayer-Zillgen et al., 2002). It is not known why OCT3 localizes to apical membranes in enterocytes but to basolateral membranes in hepatocytes. It is also not understood why OCT3 is capable of dual membrane localization that is tissue specific but OCT1

and OCT2 are restricted to basolateral membranes. Of the three OCT isoforms, only OCT3 contains a C-terminal PDZ-ligand (-RSHL). Therefore, the membrane localization of OCT3 may be determined by tissue- and domain-specific localization of PDZ proteins.

#### b. Organic Cation/Carnitine Transporters (OCTNs).

The OCTN subfamily consists of OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*). In addition to their physiological role as uptake transporters for the zwitterion carnitine, members of the OCTNs are known also to interact with drugs such as levofloxacin, oxaliplatin, and cephaloridine (Hirano et al., 2008; Jong et al., 2011; Kano et al., 2009). Analogous to OCTs, OCTNs are able to perform bidirectional transport; however there are subtle differences in the transport mechanisms between OCTN1 and OCTN2. For example, OCTN1 can function as an organic cation uniporter or an H<sup>+</sup>/organic cation antiporter, whereas OCTN2 functions as a sodium/carnitine co-transporter or may perform sodium-independent cation transport (Grundemann et al., 2005; Ohashi et al., 2001; Tamai et al., 2004; Tamai et al., 1998; Wu et al., 1999). Although broadly expressed in multiple tissues, OCTN subfamily members display preferential tissue expression. For instance, OCTN1 is predominately expressed on apical membranes of renal proximal tubules but is found at much lower levels in other tissues such as the liver, small intestine, placenta, and brain (Tamai et al., 2004; Tamai et al., 2000; Wu et al., 2000a). OCTN2 also is expressed on apical membranes of renal proximal tubule cells but its expression is not limited to polarized epithelia. It is found in several organs including the heart, brain, liver, testis, and skeletal muscle (Tamai et al., 1998; Tamai et al., 2000). Because OCTN2 is believed to be the major OCTN regulating serum carnitine concentrations (Nezu et al., 1999),

its wider expression may have evolved to ensure adequate carnitine absorption and distribution throughout the body. Carnitine is required for fatty acid transport in mitochondria for generation of metabolic energy. Underscoring its vital nature, primary carnitine deficiency, a disorder characterized by chronic muscle weakness, cardiomyopathy, hypoglycemia, and liver dysfunction, is caused by defects in OCTN2 activity (Tamai et al., 1998). The OCTN subfamily has been linked to other disease states as well. Several studies implicated OCTN1 and OCTN2 in the pathophysiology of inflammatory bowel disease, diabetes, and cancer (Martini et al., 2012; Muoio et al., 2012; Okabe et al., 2008; Peltekova et al., 2004; Pochini et al., 2013). By virtue of their association with several diseases, OCTNs are studied more in the context of pathophysiology and less for their potential pharmacological role as mediators of drug absorption and disposition. Nevertheless, OCTNs are involved in transport of organic cationic drugs and are potential sites for drug/carnitine interactions (Ganapathy et al., 2000). OCTN1 and OCTN2 mediate transport of drugs such as anti-psychotics, anti-hypertensives, antibiotics, and drugs used in the treatment of neurologic disorders (Ganapathy et al., 2000; Grube et al., 2006; Jong et al., 2011; Nakamura et al., 2010; Urban et al., 2008). Both OCTN1 and OCTN2 contain C-terminal PDZ ligands (-LTAF and -STAF, respectively). The role of PDZ proteins and OCTN membrane abundance and function is summarized in section IV.

#### c. Organic Anion Transporters (OATs).

OATs mediate the systemic removal of organic anions including drugs, toxins, and endogenous metabolites. OAT1-3 (*SLC22A6-8*), OAT4 (*SLC22A11*), and URAT1 (*SLC22A12*) are deemed clinically significant human OAT isoforms due to their prominent role in the kidney as mediators

of renal secretion and thus renal drug elimination (Burckhardt, 2012; Giacomini et al., 2010). Historically, OATs have been the targets of pharmacological strategies to extend limited supplies of drugs. The classic example of OAT-targeted interventions of this manner is the utilization of probenecid to conserve limited supplies of penicillin during World War II. Probenecid, an OAT inhibitor, increases serum concentrations of penicillin by significantly reducing OAT dependent renal secretion, thus extending the half-life of penicillin and optimizing therapeutic efficacy by achieving concentrations above the minimum inhibitory concentration with a lower dose (Weiner et al., 1960). More recently, owing to its established inhibitory effects on OATs, probenecid has been suggested as an adjuvant to other medications that have been in short supply, such as the anti-viral drug oseltamivir (Howton, 2006). Genetic variations encoding different OAT isoforms are also associated with altered transporter activity and impaired renal handling of OAT substrates (Bleasby et al., 2005; Erdman et al., 2006; Xu et al., 2005). For instance, genetic polymorphisms in OAT3 are associated with reduced renal clearance of cefotaxime, a cephalosporin antibiotic (Yee et al., 2013). Transcript variants of URAT1 may produce increases or decreases in renal tubular uric acid reabsorption, changes that result in hyperuricemia and hypouricemia respectively (Graessler et al., 2006; Wakida et al., 2005).

In contrast to the OCTs, OATs transport anions into cells against the negatively charged electrochemical gradient and therefore are energy dependent. OATs transport substrates by exchanging an intracellular mono- or divalent anion, typically a dicarboxylate, for uptake of an extracellular anion (Burckhardt, 2012). The energy expenditure that drives this process comes indirectly from the  $\text{Na}^+/\text{K}^+$ -ATPase that maintains intracellular sodium concentration at low levels and creates a negative intracellular voltage. This favorable electrochemical gradient drives sodium-coupled uptake of dicarboxylate species by the sodium-dicarboxylate co-transporter (NaDC3). Together, the  $\text{Na}^+/\text{K}^+$ -ATPase, NaDC3, and OATs cooperate to facilitate the uptake and removal of organic anions. OATs are heavily expressed in the kidney, where they play a



significant role in the uptake step of renal drug accumulation. Among the OAT family, OAT1 and OAT3 are the most extensively studied in the context of drug transport and have been shown to mediate the uptake of a wide variety of structurally diverse drug classes such as anti-hypertensive drugs, HMG Co-A-reductase inhibitors (statins),  $\beta$ -lactam antibiotics, H<sub>2</sub> receptor antagonists, anti-neoplastic drugs, non-steroidal anti-inflammatory drugs (NSAIDs), and anti-viral drugs (Burckhardt, 2012). However, OATs also transport multiple endogenous compounds and play complex physiological roles (Mandal and Mount, 2015; Nigam et al., 2015). Recent evidence suggests that OAT3 not only handles transport of drugs and drug metabolites, but also may play a role in: 1) pathways involved in the regulation of secondary metabolites, such as prostaglandins, steroids, and uric acid; 2) the metabolism and handling of gut microbiome products, as well as uremic toxins that accumulate in kidney disease; 3) handling of dietary flavonoids such as epicatechin; and 4) the tricarboxylic acid cycle and nucleotide and amino acid metabolism (Mandal and Mount, 2015; Nigam et al., 2015; Wu et al., 2013).

OAT1 and OAT3 are exclusively located at basolateral membranes in renal proximal tubule cells. In the kidney, all members of the OAT family localize to basolateral membranes with the exception of OAT4 and URAT1. OAT4 is unique in that it is expressed only in humans and is located at apical membranes of renal proximal tubule cells (Babu et al., 2002; Cha et al., 2000; Ekaratanawong et al., 2004). OAT4 is also expressed in the placenta but is restricted to basolateral membranes of syncytiotrophoblasts (Cha et al., 2000). This distinctive tissue-specific pattern of OAT4 membrane localization is similar to the dual-membrane expression patterns of OCT3. Like OCT3, OAT4 possesses a PDZ ligand (-STSL). Several studies suggest that multiple PDZ proteins may govern the membrane abundance and localization of OAT4 (Kato et al., 2004; Miyazaki et al., 2005; Zhou et al., 2008).

## 2. SLCO Family.

The *SLCO* gene family encodes the human OATPs. These proteins transport various organic amphiphilic compounds, the majority of which are large hydrophobic anions such as bile acids, steroid conjugates, and anionic peptides, as well as several drugs including statins, angiotensin II receptor antagonists and angiotensin converting-enzyme inhibitors (Babu et al., 2002; Cha et al., 2000; Ekaratanawong et al., 2004; Zhou et al., 2008). Eleven human OATPs have been identified to date. Selected OATPs discussed in this manuscript are summarized in Table 2.

There are six known human OATP families. Classification of OATPs is based on their amino acid similarities. OATPs within the same family share  $\geq 40\%$  amino acid sequence identity and are assigned root names followed by a numerical designation (OATP1-6). Subfamily members share  $\geq 60\%$  amino acid sequence identity and are designated with a letter following the numerical family designation. For instance, OATP family 2 (OATP2) has two subfamilies (OATP2A and OATP2B). A numerical designation after the subfamily heading is assigned to each member of a subfamily (OATP2A1 and OATP2B1). Size differences exist across members of the OATP family. OATPs have an average length of 710 amino acids, but range from 643 amino acids (OATP2A1) to 848 amino acids (OATP5A1). However, all OATPs share a similar predicted membrane topology that comprises 12  $\alpha$ -helical transmembrane domains, intracellular N- and C-termini, and multiple glycosylation sites within the second and fifth extracellular loop. The fifth extracellular loop is larger and contains multiple conserved cysteine residues that participate in disulfide bonds (Figure. 2). In general, OATP dependent transport involves bidirectional, sodium independent exchange. OATPs couple the cellular uptake of anions with efflux of intracellular substances such as bicarbonate, reduced glutathione, or lactate. In the case of OATP2B1, transport is pH sensitive but only with selective solutes such as statins (Kobayashi et al., 2003; Nozawa et al., 2004; Varma et al., 2011). The precise mechanism of

transport and how it relates to the physiological and pharmacological role of individual OATPs remains unclear.

Tissue expression and membrane localization of selected *SLCO* drug transporters is summarized in Figure 1. Some members of the OATP family display broad tissue expression. OATP1A2, OATP2A1, OATP2B1, OATP3A1, and OATP4A1 for instance are found in multiple tissues including the kidney, placenta, skeletal muscle, choroid plexus, and endothelial cells of the blood-brain-barrier. Other OATPs exhibit a more restricted pattern. The major hepatic OATP isoforms include OATP1B1 and OATP1B3; OATP4C1 predominates in the kidney. Among the OATP family, OATP1B1, OATP1B3, OATP1A2, and OATP2B1 are considered clinically important determinants of pharmacokinetics due to their participation in transporting commonly used medications such as statins (Giacomini et al., 2010). In particular, OATP1B1 and OATP1B3 mediate the hepatic uptake of drugs whereas OATP1A2 and OATP2B1 mediate intestinal drug absorption. The extensive expression of OATPs in the intestine and liver likely evolved as a protective mechanism to combat inadvertent ingestion of plants or foods containing toxins. An additional evolutionary advantage to OATP dependent detoxification comes in the form of cooperativity with intestinal and hepatic metabolizing enzymes. As a means to provide protection against a wide spectrum of potentially harmful exogenous substances, intestinal and hepatic OATPs have adopted overlapping substrate specificity with intracellular cytochrome P450 (CYP) enzymes. Together, OATPs cooperate with CYP enzymes in the intestine and liver to reduce the systemic exposure of xenobiotics by exposing substrates to intracellular CYPs for subsequent metabolism. Evidence of this cooperative functionality is observed concerning the hepatic clearance of erythromycin, a commonly used probe to assess CYP3A function (Rivory et al., 2001). Erythromycin uptake into hepatocytes is mediated by OATPs (Lancaster et al., 2012; Sun et al., 2010). OATPs facilitate the uptake of erythromycin from the portal vein and into hepatocytes where metabolism by CYP3A occurs. (Franke et al., 2011). Therefore, hepatic clearance of erythromycin is a function of OATP-dependent uptake

and CYP3A function. Although these examples illustrate OATP-CYP cooperativity, they may not extend to other transporters and CYP proteins.

Although generally considered drug transporters, physiological roles of OATPs also have been described. Members of the OATP family are involved in the transport of bilirubin (OATP1B1 and OATP1B3) and thyroid hormones (OATP1C1) (Keppler, 2014; Mayerl et al., 2014). In the liver, bilirubin metabolism and elimination is accomplished, in part, by the coordinated actions of OATPs and UDP glucuronosyltransferase-1 family, polypeptide-1 (UGT1A1). Similar to the proposed mechanism of OATP-CYP cooperative functionality and drug metabolism, hepatic OATP1B1 and OATP1B3 facilitate the uptake of unconjugated bilirubin, which is followed by glucuronic acid conjugation catalyzed by intracellular UGT1A1 (Keppler, 2014). OATPs also may be involved in transporting endogenous substances that are otherwise impermeant and cannot traverse the cell membrane passively and, therefore, rely on transporters to enter cells. OATP1C1 has been implicated recently in the transport across the blood-brain barrier of thyroxine ( $T_4$ ), the prohormone of triiodothyronine ( $T_3$ ), a process thought to be important for proper brain development (Mayerl et al., 2014). The physiological and pharmacological importance of renal OATPs, particularly the role of OATP4C1, is not fully appreciated.

Similar to other SLC transporter families, membrane localization of OATP isoforms is not uniform and in some cases is tissue dependent. Although most are located on basolateral membranes, OATP1A2 is exclusive to apical membranes, whereas OATP2B1 displays dual membrane localization. In the intestine, OATP1A2 and OATP2B1 are located on the apical brush-border membrane, where they contribute to drug absorption. In the liver, OATP1B1, OATP1B3, and OATP2B1 are located on basolateral/sinusoidal membranes, where they may be responsible for hepatic drug uptake (Kobayashi et al., 2003; Kullak-Ublick et al., 2001; Tamai, 2012). OATP1A2 is also found at apical membranes in endothelial cells of the blood-brain barrier, hepatocytes, and renal distal tubules (Lee et al., 2005). Analogous to the

OCT and OAT families of drug transporters, some OATP isoforms harbor a C-terminal PDZ ligand and are binding partners with PDZ proteins (Kato et al., 2004; Wang et al., 2005; Zheng et al., 2014). Multiple PDZ proteins regulate the function of OATP1A2 (Zheng et al., 2014), which is discussed in section IV.

The OATP family is gaining considerable attention due to their central role in drug disposition and response (Group et al., 2008; Shitara et al., 2013). This recognition is underscored by the importance of OATPs on statin pharmacotherapy. Statins are orally administrated medications and considered mainstay therapy for the treatment of hypercholesterolemia. In order to elicit their lipid-lowering effects, statins must be taken up into hepatocytes. Thus, OATPs are not only critical determinants of statin pharmacokinetics, but also for delivery to their site of action. Several OATPs are involved in the oral absorption and disposition of statins, particularly OATP1B1 (Hirano et al., 2006; Prueksaritanont et al., 2014; Romaine et al., 2010). All statins in clinical use are substrates for OATP1B1 (Niemi et al., 2011). Further, OATP1B1 polymorphisms have been shown to alter the pharmacokinetics and pharmacodynamics of statins including pravastatin, atorvastatin, and simvastatin (Meyer Zu Schwabedissen et al., 2015; Oh et al., 2013). OATP1A2 has also been recognized as important for drug transport and disposition. Substrates for OATP1A2 include statins, HIV-protease inhibitors and fexofenadine (Kullak-Ublick et al., 1998). Selected OATP substrates are listed in Table 2. By virtue of their expanding role in pharmacokinetics and pharmacodynamics, characterizing OATP regulatory mechanisms in health and disease remains an area of focus

### 3. Other SLCs (SLC15A Family).

The *SLC15A* gene family encodes two human peptide transporters, PEPT1 (*SLC15A1*) and PEPT2 (*SLC15A2*). PEPT1 and PEPT2 function primarily as intestinal and renal peptide transporters, respectively. Both PEPT1 and PEPT2 are sodium independent symporters that couple peptide transport with the movement of  $H^+$  down its electrochemical gradient. PEPT1 is a high-capacity low-affinity transporter for di- and tripeptides but has negligible affinity for amino acids (Liang et al., 1995). PEPT1 is heavily expressed in the small intestine, where it localizes exclusively to the apical brush-border membranes of enterocytes (Liang et al., 1995). At the intestinal barrier, PEPT1 absorbs small peptides that are generated upon enzymatic breakdown of endogenous or ingested protein. PEPT1 also plays an important pharmacological role as a mediator of oral drug absorption and is a target for pro-drug design (Brandsch, 2013; Hamman et al., 2005; Zhang et al., 2013). Hydrophilic drug compounds that are taken orally often have poor bioavailability secondary to limited intestinal membrane permeability. This barrier to pharmacotherapy and limitation to drug design has been circumvented by exploiting intestinal PEPT1. As a strategy to enhance the oral bioavailability of such compounds, pro-drugs are formulated via amino acid esterification (Majumdar and Mitra, 2006). Chemical modifications of this type thus permit involvement of PEPT1 in drug uptake processes in the intestine. This approach has been used previously, most notably in the design of the nucleoside analog valacyclovir (Jacobson, 1993). Valacyclovir, a PEPT1 substrate and amino acid ester derivative of the active drug acyclovir, exhibits a three to five fold increase in bioavailability (Weller et al., 1993). Intestinal PEPT1 is also involved in the absorption of other peptidomimetic drug compounds and pro-drugs including  $\beta$ -lactam antibiotics and angiotensin converting-enzyme inhibitors (Daniel and Adibi, 1993; Ganapathy et al., 1995; Shu et al., 2001; Sugawara et al., 2000). PEPT2 is predominately expressed in renal proximal tubules and localizes to apical

membranes, where it is involved in reabsorption of di- and tripeptides from the glomerular filtrate (Rubio-Aliaga et al., 2003). PEPT1 is also found at apical membranes in proximal tubules, where it may be involved in the reabsorption of peptides that are generated by the action of brush border peptidases on filtered proteins. Although both PEPT isoforms are expressed in kidney, PEPT1 is expressed at much lower levels, and therefore the relative contribution of PEPT1 to renal tubule reabsorption is likely less than that of PEPT2 (Liang et al., 1995). Further, in contrast to PEPT1, PEPT2 is a high-affinity low-capacity transporter. This clear distinction in transport kinetics was described by revealing that PEPT1 and PEPT2 were responsible for the low-affinity ( $K_m$  of 1.2 mM) and high-affinity ( $K_m$  of 50  $\mu$ M) transport of glycylsarcosine (Gly-Sar) respectively (Takahashi et al., 1998). It was later determined in studies comparing the contribution of PEPT1 and PEPT2 to the renal reabsorption of  $\beta$ -lactam antibiotics, that PEPT2 mediates high-affinity transport of amino  $\beta$ -lactam antibiotics such as amoxicillin, cephalexin, and cefadroxil, which contain an  $\alpha$ -amino group, and that the involvement of PEPT1 is minimal in this regard (Inui et al., 2000; Takahashi et al., 1998). These findings strongly support that the pharmacological role of PEPT2 in the kidney might be to participate in reabsorption of amino  $\beta$ -lactam antibiotics, as well as other pharmacologically active compounds with similar chemical structures. Taken together, the expression pattern and transport kinetics of the PEPTs are not surprising if consideration is given to their physiological roles. The abundant expression of PEPT1, a high-capacity transporter, at the apical membrane in enterocytes is strategically placed to ensure adequate dietary protein absorption. Conversely, the expression of PEPT2, a high-affinity transporter with specific substrate affinities, at apical membranes in renal proximal tubules cells facilitates tight control of compounds that undergo renal elimination. The pharmacological implications of PEPT1 and PEPT2 function may be to mediate the intestinal absorption and renal reabsorption of drugs respectively.

Both PEPT isoforms diverge in their substrate affinity and transport capacity but share substrate specificity, transport mechanisms, and are exclusively located at apical membranes (Daniel and Adibi, 1993; Ganapathy et al., 1995; Leibach and Ganapathy, 1996; Liang et al., 1995; Lin et al., 1999; Saito et al., 1996). The mechanism for the exclusive apical localization of the PEPTs in the intestine and kidney is not clear. However, PEPT1 (-QKQM) contains a class III PDZ ligand whereas PEPT2 (-KTKL) contains a class I PDZ-ligand and both PEPT1 and PEPT2 interact with PDZ proteins (Kato et al., 2004). Localization of PEPT1 at apical membranes in enterocytes from *nherf3*<sup>-/-</sup> mice is almost completely absent indicating NHERF3 may play a role in PEPT1 regulation (Sugiura et al., 2008). Although multiple PDZ proteins have been shown to influence PEPT2 function, the role of PDZ proteins on PEPT1 function is less understood (Boehmer et al., 2008; Kato et al., 2004; Noshiro et al., 2006). These observations suggest that PDZ regulation of drug transporter membrane abundance may not solely depend on the presence of a class I PDZ-ligand. This may indicate that PDZ-drug transporter interactions are mediated through other binding motifs or facilitated by other cytoplasmic adapter proteins. A detailed analysis on the differences in PDZ regulation of PEPT1 and PEPT2 in the intestine and kidney respectively, will address this disparity and may provide important clues leading to the complexity of PDZ function.

## B. ATP Binding-Cassette (ABC) Drug Transporters

The ABC superfamily represents the largest family of membrane transporters, comprising 49 members divided into 7 subfamilies (*ABCA-ABCG*) (Table 2). Members of subfamilies B, C, and G are the best studied in the context of drug transport and disposition. Specifically, the multidrug



resistance protein (MDR1, *ABCB1*), multidrug resistance-associated proteins (MRPs, *ABCCs*), and breast cancer resistance protein (BCRP, *ABCG2*) are clinically significant mediators of multi-drug resistance and response (Giacomini et al., 2010; Hillgren et al., 2013; Zamek- Gliszczyński et al., 2012). Drug resistance is a clinical problem in the treatment of many types of cancer. Resistance to several anti-cancer agents is caused, in part, by increased ABC drug transporter function (Kathawala et al., 2014). In tumors, ABC drug transporters actively extrude anti-cancer agents and prevent intracellular drug accumulation resulting in poor drug response (Kim et al., 2014). In a similar manner, ABC drug transporters limit the systemic exposure of drugs and drug metabolites by preventing drug absorption and promoting drug elimination at anatomical barriers such as intestinal, hepatic, and renal epithelia, tissues where ABC drug transporter expression is high. The mechanism of transport is similar among all members in that they require ATP hydrolysis to energize cellular drug efflux. Most members share a membrane topology consisting of 12 hydrophobic TMDs that are divided into two distinct clusters, each containing six TMDs, and each with its own nucleotide binding-domain (NBD) (Figure. 3). MRP1, MRP2, MRP3, MRP6, and MRP7 harbor an additional cluster consisting of five TMDs near the N-terminus (Figure. 3.) (Choi and Yu, 2014). BCRP is unique among the ABC family in that it contains only six TMDs and one NBD (Figure. 3). For that reason BCRP is considered a “half-transporter.” ABC drug transporters expressed in the intestine, liver, and kidney mediate the cellular efflux of a vast array of structurally diverse drugs from several drug classes including, anti-cancer drugs, antibiotics, statins, and HIV protease inhibitors, among many others (Cascorbi, 2011; Keppler, 2011; Mao and Unadkat, 2014). ABC drug transporters are also the molecular basis for clinically significant drug-drug interactions, which lead to changes in serum drug concentrations and consequently drug response (Konig et al., 2013). In order to limit drug exposure by promoting drug elimination in polarized epithelia, ABC drug transporters must be targeted to the membrane domain that complements this function, which in many cases is the apical membrane facing a luminal space. ABC drug transporters facilitate drug extrusion into

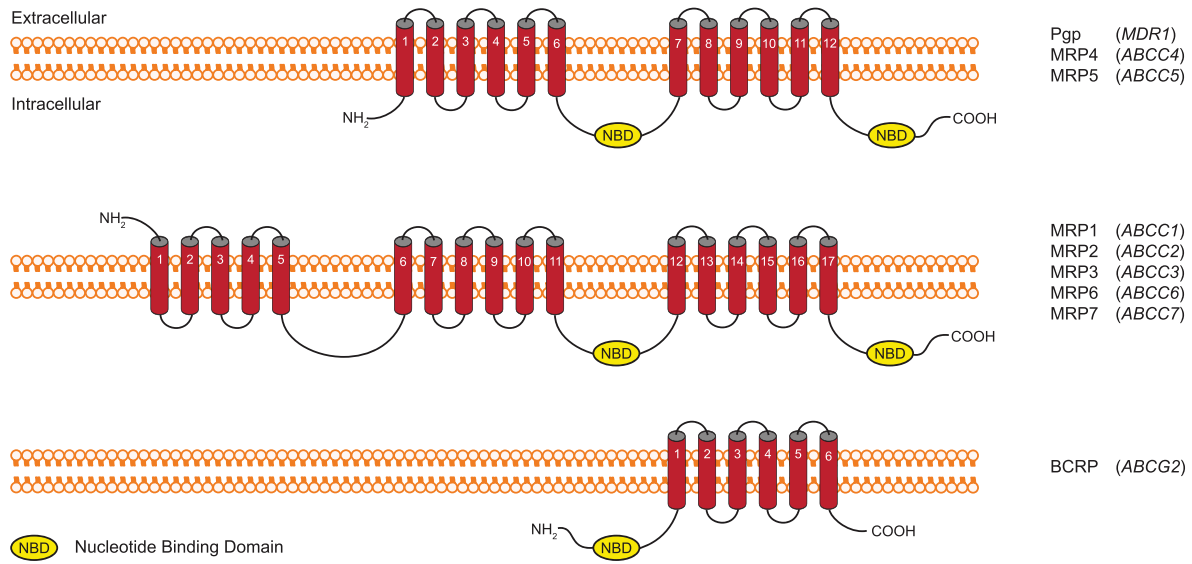


Figure 3

bile (from hepatocytes), urine (from renal proximal tubule cells), and the intestinal lumen (from enterocytes). Tissue expression and membrane localization of selected ABC drug transporters is summarized in Figure 1. ABC drug transporters and a list of selected substrates are summarized in Table 2.

## 1. MDR1 (ABCB1).

The *ABCB1* gene encodes MDR1, commonly referred to as P-glycoprotein (Pgp). Pgp was one of the first members of ABC drug transporters to be identified and has been well studied since it was first cloned and characterized nearly four decades ago (Juliano and Ling, 1976). Pgp is extensively distributed throughout the body with highest expression in epithelial cells with excretory roles such as intestinal, hepatic, and renal epithelia (Thiebaut et al., 1987). It is also expressed in epithelial cells lining the pancreatic ducts, as well as in endothelial cells of the blood-brain barrier (Beaulieu et al., 1997; Thiebaut et al., 1987). Pgp specifically localizes to apical membranes. The location of Pgp at the luminal surface supports its physiological role to protect susceptible organs from a broad array of exogenous compounds and environmental toxins including an equally diverse list of drugs and drug metabolites (Padowski and Pollack, 2010; Staud et al., 2010). Pgp actively extrudes intracellular substrates in an ATP-dependent manner and prevents tissue accumulation by promoting excretion. Pgp substrates include multiple drugs from structurally diverse classes such as immunosuppressants, antibiotics, statins,  $\beta$ -blockers, anti-cancer drugs, and the cardiac glycoside digoxin, among many others (Cascorbi, 2011; Cascorbi and Haenisch, 2010; Fromm, 2004).

Pgp is limited to apical membranes. The mechanism of its specific membrane domain localization is unknown. Pgp does not harbor a C-terminal PDZ ligand and there is no evidence for interaction with PDZ proteins. Its homolog, the cholesterol efflux regulatory protein (*ABCA1*), contains a PDZ ligand (-ESYV) and is regulated by PDZ proteins (Okuhira et al., 2010). Though Pgp appears to not interact with PDZ proteins directly, radixin, a cytoskeletal adapter protein that interacts with PDZ proteins, plays an important role in regulating intestinal Pgp localization and function (Yano et al., 2013). This implies that PDZ proteins may be involved in regulating Pgp localization indirectly *via* interactions with radixin or other cytoskeletal adapter proteins such as ezrin or moesin. Regulation of Pgp function secondary to membrane abundance and localization is not well studied and may be another example of higher order complexity regarding PDZ function.

Pgp is widely acknowledged as a clinically significant mediator of pharmacokinetics and pharmacodynamics due to its high expression in tissues important for drug disposition and well-established list of substrates and inhibitors (Giacomini et al., 2010). Pgp is implicated in several clinically significant drug-drug interactions. The quindine-digoxin interaction is a classic example of a Pgp mediated drug-drug interaction (Bigger and Leahey, 1982). The anti-arrhythmic drug quinidine is an established Pgp inhibitor. When co-administered with the cardiac glycoside digoxin, a Pgp substrate, patients are at risk for digoxin-induced toxicity including the development of cardiac arrhythmias (Gessman et al., 1983). This adverse drug event is partially attributed to an increase in the systemic exposure of digoxin secondary to impaired Pgp dependent renal elimination (De Lannoy et al., 1992). Other clinically significant Pgp interactions have been documented (Aszalos, 2007). For these reasons, regulatory agencies such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA) suggest that all new drug candidates be screened for *in-vitro* Pgp substrate and inhibition liability.

## 2. MRPs (ABCCs)

The MRP family of drug transporters is encoded by the ABCC gene subfamily. The MRP family consists of 9 proteins (MRP1-9). Analogous to other members of the ABC superfamily of drug transporters, MRPs mediate cellular drug efflux and play a central role in protecting tissues from accumulation of toxic compounds. MRPs are also responsible for transporting several endogenous physiological molecules, illustrating that their function is not purely pharmacological. For example, MRP1 mediates the transport of reduced and oxidized glutathione and the pro-inflammatory mediator, cysteinyl leukotriene C<sub>4</sub> (Cole, 2014). The physiological role of MRP4 is to regulate intracellular concentrations of cyclic nucleotides, such as cAMP and cGMP, by functioning as a nucleotide efflux transporter. MRP4 regulation of cAMP has recently been implicated in the mechanism of drug-induced diarrhea, and in fibroblast cell migration (Moon et al., 2015; Sinha et al., 2013). In addition to their pharmacological role as mediators of drug disposition, dysfunctional MRPs are responsible for the development of pathophysiological conditions. For example, sequence variations that result in loss of functional MRP2 and MRP6 are the molecular basis for Dubin-Johnson syndrome (DJS) and pseudoxanthoma elasticum (PXE), respectively (Bergen et al., 2007; Kartenbeck et al., 1996; Toh et al., 1999). DJS is characterized by dysfunctional hepatic secretion of conjugated bilirubin into the bile. The loss of functional MRP2 on the apical/canalicular membrane of hepatocytes impairs biliary excretion of conjugated bilirubin, which results in elevated serum concentrations of bilirubin and the development of DJS (Devgun et al., 2012). PXE is characterized by mineralization and eventual degradation of elastic fibers in connective tissue, which can lead to loss of vascular tone and premature arteriosclerosis (Li et al., 2009). Although the molecular pathologies of PXE are still unclear, mutations in MRP6 cause PXE (Bergen et al., 2007). The physiological MRP6 substrates that are involved in the development of PXE remain unknown. In

accordance with their functional role, MRPs are prominently expressed at anatomical barriers, with high expression in the intestine, liver, kidney, and blood-brain barrier.

The MRPs display overlapping substrate specificity for many endogenous and exogenous lipophilic organic anions. MRP drug substrates include statins, angiotensin II receptor antagonists, anti-viral drugs, and chemotherapeutic agents (Keppler, 2011). Within the MRP family, MRP2 and MRP4 are deemed the most clinically significant to drug development due to their established influence on drug disposition and expanding list of drug substrates (Giacomini et al., 2010; Hillgren et al., 2013; Zamek-Gliszczynski et al., 2012). Several commonly used medications are substrates for MRP2 dependent transport including methotrexate, olmesartan, and etoposide (Gerk and Vore, 2002). In the kidney, MRP4 plays a principal role in the renal luminal efflux of acyclic nucleotide reverse transcriptase inhibitors such as adefovir and tenofovir (Imaoka et al., 2007). An important clinical limitation to the usage of adefovir and tenofovir is the development of nephrotoxicity, which has been proposed to be partially attributable to a reduction in MRP4 dependent renal secretion (Izzedine et al., 2005). MRP2 and MRP4 seem to be responsible for drug transport, whereas other members of the MRP family are involved in transport of endogenous substrates. Although both MRP2 and MRP4 are expressed in the liver and kidney, MRP2 is considered the major hepatic isoform whereas MRP4 predominates in the kidney. Interestingly, both hepatic and renal MRP2 localize to apical membranes, whereas kidney MRP4 is found at apical membranes but at basolateral surfaces in hepatocytes. The mechanism responsible for the difference in membrane localization of MRP4 in the liver *versus* the kidney is not known. Both MRP2 (-STKF) and MRP4 (-ETAL) contain C-terminal PDZ ligands. Multiple PDZ proteins regulate MRP2 and MRP4 function secondary to preservation of membrane abundance and organization of membrane localization (Hoque et al., 2009; Karvar et al., 2014; Park et al., 2014). In addition, members of the ERM family, such as radixin and ezrin, have been shown to regulate MRP2 function. Radixin selectively modulates the functional expression of hepatic MRP2, but not other MRP isoforms

(He et al., 2012; Kikuchi et al., 2002). Other studies show that both radixin and ezrin are required for MRP2 apical membrane localization (Yang et al., 2007). The role of radixin and ezrin in targeting MRP2 to apical membranes is believed to be secondary to preventing internalization (Kojima et al., 2008; Rost et al., 2008; Saeki et al., 2011; Yang et al., 2007). Considering PDZ proteins are known to interact with the ERM family, these observations may indicate that MRPs are subject to tissue specific PDZ regulation dictated by distinct patterns of tissue expression and co-localization of PDZ proteins with selected ERM proteins. Therefore, tissue-specific ERM proteins may influence the non-redundant physiological and pharmacological roles of PDZ proteins in different tissues. Determining how PDZ proteins cooperate with cytoskeletal adapter proteins to regulate membrane abundance, localization, and function of target proteins in different tissues, such as drug transporters, may address these issues.

### 3. BCRP (ABCG2).

Breast cancer resistance protein (BCRP) is the second member of the G subfamily of ABC drug transporters and is encoded by *ABCG2*. BCRP was originally identified in several breast cancer cell lines, where it confers resistance to multiple anti-cancer drugs. (Dietel et al., 1990; Futscher et al., 1994; Kellner et al., 1997; Nakagawa et al., 1992; Taylor et al., 1991). It was later cloned, characterized, and assigned to the G subfamily of ABC drug transporters (Doyle et al., 1998). BCRP contains only 6 TMDs and one NBD and thus is considered a half transporter because it diverges from the prototypical membrane topology of other ABC drug transporters. Recent evidence shows that BCRP forms homodimers or homooligomers in intact cells (Ni et al., 2010).

Covalent linkages mediated by cysteine residues in the third extracellular loop are believed to be responsible for BCRP dimerization (Shigeta et al., 2010). However, dimerization is not a requirement for BCRP function (Kage et al., 2005; Shigeta et al., 2010). The expression pattern of BCRP complements its role as an efflux drug transporter. BCRP is highly expressed at the luminal surface of intestinal and hepatic epithelia where it attenuates intestinal drug absorption and promotes biliary drug excretion (Hillgren et al., 2013). The highest expression of BCRP is detected in placental tissue, specifically on apical syncytiotrophoblast membranes. Other tissues with high expression of BCRP include the blood-brain barrier, testis, and lactating mammary tissue (Jani et al., 2014). In the kidney, BCRP localizes to apical membranes of proximal tubule epithelial cells, however its level of expression is less compared to the intestine and liver (Mao and Unadkat, 2014). BCRP transports a broad array of both endogenous and exogenous compounds, including several marketed drugs such as anti-cancer agents, nucleoside analogs, and statins. A list of selected BCRP substrates is summarized in Table 2.

Recent clinical pharmacogenetic studies have emphasized the importance of BCRP to drug disposition and response, which has prompted the FDA and EMA to recommend that all investigational new drugs be tested for BCRP substrate and inhibitor liability, except for substrates that fall into biopharmaceutical classification system 1; drugs with high solubility and high permeability. Loss of function BCRP polymorphisms that result in decreased drug clearance and increased drug exposure and toxicity have been reported. For example, in carriers of BCRP polymorphisms, the systemic exposure of several drugs such as atorvastatin, rosuvastatin, sunitinib, and sulfasalazine is significantly increased (Giacomini et al., 2013; Lee et al., 2015; Mizuno et al., 2012). In the case of sunitinib, carriers of BCRP polymorphisms also experience a higher incidence of sunitinib-induced toxicity (Mizuno et al., 2012). In addition to altering pharmacokinetics and pharmacodynamics, BCRP polymorphisms are associated with disease states such as Alzheimer's disease and gout (Feher et al., 2013; Matsuo et al., 2011b; Takada et al., 2014). The association between BCRP and these disease states is hypothesized



to be secondary to BCRP dependent handling of amyloid beta in Alzheimer's disease and uric acid in patients with gout (Abuznait and Kaddoumi, 2012; Matsuo et al., 2011a; Matsuo et al., 2011b).

BCRP membrane location in polarized cells is restricted to apical membranes. The mechanism that underlies the restricted apical membrane localization of BCRP has yet to be defined. Despite the fact that BCRP (-KKYS) does not harbor a PDZ ligand, NHERF3 regulates intestinal BCRP function and plays an important role in localizing BCRP to apical membranes in enterocytes (Shimizu et al., 2011). NHERF3 also regulates intestinal PEPT1, which harbors a class III PDZ ligand (-QKQM) (Shimizu et al., 2011; Sugiura et al., 2008). These observations suggest that the interaction between NHERF3 and BCRP differs from those of other drug transporters, which interact with NHERFs *via* a canonical PDZ ligand. The mechanism of NHERF3 regulation of BCRP may involve the presence of a non-canonical PDZ-binding motif. The NHERF3-interacting binding sequence in BCRP is unclear. Due to its extensive tissue distribution, expanding list of drug substrates, and association with altered pharmacokinetics and pathophysiology, BCRP has been recognized as a clinically relevant drug transporter of emerging importance.

### III. TISSUE AND CELLULAR LOCALIZATION OF PDZ PROTEINS

PDZ domains are the most abundant protein-protein interaction modules in humans (Feng and Zhang, 2009; Ponting et al., 1997). Canonical PDZ domains consist of 80 to 90 amino acid residues that form six  $\beta$ -stands ( $\beta$ A to  $\beta$ F) and two  $\alpha$ -helices ( $\alpha$ A and  $\alpha$ B) that are arranged in a three-dimensional globular structure (Karthikeyan et al., 2001). Binding of a protein ligand to the PDZ domain occurs within a hydrophobic binding pocket that is created by the  $\beta$ B strand, the  $\alpha$ A helix, and the loop connecting the  $\alpha$ A and  $\beta$ B strands. This carboxylate-binding loop contains a GLGF core motif or a related sequence such as GYGF, as is the case with the NHERF family of PDZ proteins (Ponting et al., 1997). PDZ domains interact with target proteins *via* specific internal or carboxy-terminal amino acid residues, commonly referred to as PDZ ligands. Carboxy-terminal PDZ ligands are a short stretch of amino acids approximately three to four residues in length, though upstream sequences affect affinity and specificity of binding. The amino acid sequence that comprises the PDZ ligand is conventionally numbered starting from the last amino acid at the extreme carboxy-terminus and is assigned position zero (P0).

PDZ recognition motifs are grouped into three classes (Class I-III) according to the consensus sequence of the PDZ ligand for which they bind. Table 1 summarizes the classification of PDZ ligands. Binding specificity among the three classes is determined, in part, by the interaction between the first amino acid residue in the  $\beta$ B-helix of the PDZ domain and the amino acid at position two (P2) of the PDZ ligand in the target protein. There are over 150 PDZ domain-containing proteins and more than 250 distinct PDZ domains in the human proteome. Individual PDZ proteins may contain multiple PDZ domains as well as other

interaction modules such as ezrin-binding domains (EBDs). PDZ proteins lack intrinsic catalytic activity and therefore function primarily as scaffold proteins, where they execute a range of biological functions that include establishing and maintaining cellular polarity, assembling multi-protein signaling complexes, and anchoring transmembrane proteins, including transporters, to the actin cytoskeleton via interactions with the ezrin/radixin/moesin/merlin (ERM) family of adapters (Georgescu et al., 2014; Ponting et al., 1997; Wang et al., 2010; Wang et al., 2007; Wang et al., 2012; Zheng et al., 2014).

NHERF proteins are the most well studied PDZ proteins that are expressed in polarized epithelia. The NHERF family consists of four structurally related proteins (NHERF1-4) (Figure. 4). NHERF1 and NHERF2 contain two tandem non-identical PDZ domains and an EBD, whereas NHERF3 and NHERF4 have four PDZ domains but lack an EBD (Seidler et al., 2009). NHERF proteins play an important role in organizing signaling complexes, controlling apical membrane trafficking, and coupling apical membrane transporters and receptors with other PDZ targets (Georgescu et al., 2014; Klenk et al., 2010; Wang et al., 2007). Although NHERF isoforms share these common functional characteristics, they perform distinct physiological functions. For example, both NHERF1 and NHERF3 are important for phosphate homeostasis, yet in *nherf1*<sup>-/-</sup> and *nherf3*<sup>-/-</sup> mice, only *nherf1*<sup>-/-</sup> mice exhibit prominent phosphate wasting, suggesting that NHERF1 and NHERF3 play different physiologic roles (Giral et al., 2011; Kocher et al., 2003; Shenolikar et al., 2002). NHERF proteins also exhibit differential tissue expression. NHERF1 and NHERF2, for instance, are abundantly expressed and widely distributed, with NHERF2 displaying a more restricted tissue distribution compared to NHERF1. NHERF1 is highly expressed on the apical aspect of polarized epithelia in the kidney, liver, and placenta (Reczek et al., 1997). NHERF1 also is found in regions of the gastrointestinal tract (small intestine and colon), gastric parietal cells, and brain (Reczek et al., 1997; Weinman et al., 1995). Although NHERF2 is co-expressed with NHERF1 in the kidney, its distribution within the kidney differs. NHERF1 is found in proximal tubules, whereas NHERF2 is expressed in the

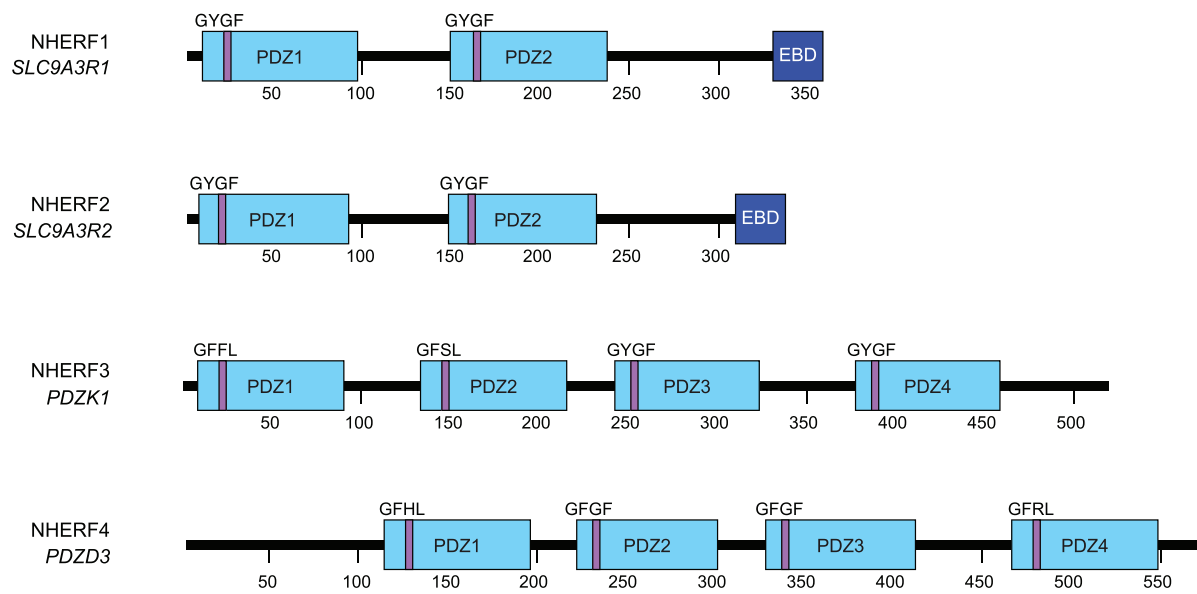


Figure 4

glomeruli, collecting duct, and throughout the renal vasculature and is absent from the proximal tubule. The highest expression of NHERF2 is in lung, including pulmonary alveoli. NHERF3 is expressed at the brush-border membrane of proximal tubule cells, the intestinal epithelium, and is also found in the liver (Custer et al., 1997; Gisler et al., 2001; Kocher et al., 1998). NHERF1 and NHERF3 predominantly localize to the apical aspect of polarized epithelia, whereas the localization of NHERF2 is variable including both apical membranes and a diffuse sub-cellular cytosolic distribution among different tissues. NHERF4 represents the isoform with the most restricted tissue distribution. Significant levels of NHERF4 are found in the kidney and gastrointestinal tract. Within these tissues, NHERF4 is localized to the apical surface and sub-apical regions throughout the cytoplasm (Donowitz et al., 2005; Gisler et al., 2001).

NHERF proteins can form homo- and heterodimers or undergo oligomerization. Dimerization is thought to be mediated either by intermolecular interactions between PDZ domains or by an intramolecular head-to-tail interaction between the carboxy-terminus and a PDZ domain within the same protein, as is the case with NHERF1 (Lau and Hall, 2001; Morales et al., 2007; Shenolikar et al., 2001). Head-to-tail dimerization of NHERF1 is mediated by the interaction between the carboxy-terminus, which itself serves as a PDZ-ligand (-FSNL), with PDZ2 (Morales et al., 2007; Wang et al., 2012). Although the biological significance of NHERF dimerization is not fully appreciated, a recent study showed that heterodimerization of NHERF2 and NHERF3 is required for the inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger-3 by carbachol (Yang et al., 2014). The formation of a large scaffolding complex comprising multiple NHERF isoforms because of dimerization may give rise to an elaborate network of cytoplasmic scaffold proteins. Formation of such a complex may dictate unique tissue-specific NHERF function. Elucidating the physiological importance of NHERF dimerization as it relates to function may aid in our understanding of the *in-vivo* roles of the NHERF family.

Despite the structural similarities in PDZ domains among the NHERF family, target proteins that harbor PDZ ligands, including drug transporters, display different affinities for

NHERF isoforms (Hoque and Cole, 2008; Park et al., 2014; Wang et al., 2010). Such differences in affinity may partially explain the non-redundant physiological functions of the NHERF family, as well as the fact that several tissues express multiple NHERF isoforms (Voltz et al., 2001). Additionally, due to the fact that NHERF proteins can form homo- and heterodimers, some NHERF isoforms may operate independently, whereas other physiological processes may require NHERF cooperativity. In the latter scenario, NHERF proteins would bind to target proteins directly *via* one PDZ domain, reserving the remaining PDZ domain(s) to interact with other NHERF isoforms that in turn are responsible for anchoring the entire multi-protein complex to the actin cytoskeleton through interactions with the ERM family. Further studies are needed to determine the physiological and potential pharmacological relevance of NHERF dimerization, cooperativity, and regulation of drug transport.

Given that NHERF expression parallels that of drug transporters and that several drug transporters have been identified as NHERF binding partners, these proteins constitute a novel class of post-translational mediators of drug transport. For this reason, among all PDZ proteins, the NHERF proteins are the most extensively studied in the context of drug transporter regulation.

#### IV. ROLE OF PDZ PROTEINS IN REGULATION OF CELL MEMBRANE ABUNDANCE, LOCALIZATION, AND FUNCTION OF DRUG TRANSPORTERS

The widely acknowledged clinical significance of drug transporters has led to a surge in studying their regulatory mechanisms. Recently, the mechanisms that underlie drug transporter membrane organization and abundance has gained considerable interest. In order to coordinate the vectorial movement of drugs across polarized epithelia, drug transporters must be sorted and stabilized to the appropriate membrane domain. By virtue of the fact that drug transporters display unique tissue-specific apical or basolateral membrane localization, domain organization is not random. If the mechanisms that dictate membrane organization and stability are faulty, drug transporters may localize to the wrong membrane domain or display decreased membrane abundance. The consequences of faulty membrane targeting could result in an overall reduction in transporter function, which may then alter the pharmacokinetics and pharmacodynamics of drugs that utilize transporter pathways for elimination. The mechanism of NHERF1 dependent renal phosphate excretion, secondary to its role in stabilizing the sodium phosphate co-transporter-2a (NaPi-2a) to the apical membrane in renal proximal tubule cells, best illustrates this. In the absence of NHERF1, the membrane abundance of NaPi-2a decreases, preventing phosphate reabsorption from the glomerular filtrate and thus promoting renal phosphate excretion (Shenolikar et al., 2002). Exploring NHERF in the setting of drug transport may address disparities surrounding drug transporter function and drug response. Emerging evidence has shown that PDZ proteins, namely the NHERF family, play a critical role in the localization, stabilization, and functional regulation of select SLC and ABC drug transporters (Table 3) (Sugiura et al., 2011).

TABLE 3

## NHERF-dependent regulation of drug transporter function

Transporter	NHERF Isoform	Experimental Model	Substrate	Transporter Function	Citation
OCTN2	NHERF3 NHERF4	HEK293	Carnitine	Increase	(Kato et al., 2005) (Watanabe et al., 2006)
Octn2	Nherf3	<i>Nherf3</i> <sup>-/-</sup> mice	Carnitine	Decrease	(Sugiura et al., 2008)
OAT4	NHERF1 NHERF3	HEK293 LLC-PK1	Estrone-3-Sulfate	Increase	(Miyazaki et al., 2005) (Zhou et al., 2008)
URAT1	NHERF3	HEK293	Urate	Increase	(Anzai et al., 2004)
OATP1A2	NHERF1 NHERF3	HEK293	Estrone-3-Sulfate	Increase	(Zheng et al., 2014)
Oatp1a1	Nherf3	<i>Nherf3</i> <sup>-/-</sup> mice	Estrone-3-Sulfate	Increase	(Sugiura et al., 2010)
PEPT2	NHERF2	<i>Xenopus</i> oocytes	Gly-Gly	Increase	(Boehmer et al., 2008)
PEPT2	NHERF3	HEK293	Gly-Sar	Increase	(Noshiro et al., 2006) (Sugiura et al., 2006)
Pept1	Nherf3	<i>Nherf3</i> <sup>-/-</sup> mice	Cephalexin	Decrease	(Sugiura et al., 2008)
MRP2	NHERF1	WIF-B	CMFDA	Increase	(Karvar et al., 2014)
MRP4	NHERF1	HeLa	6-mercaptopurine, 9-[2-(phosphonylmethoxy) ethyl]-adenine	Decrease	(Hoque and Cole, 2008)
MRP4	NHERF3	HEK293	Adefovir	Increase	(Park et al., 2014)
Mrp4	Nherf3	<i>Nherf3</i> <sup>-/-</sup> mice	Adefovir	Decrease	(Park et al., 2014)
Bcrp	Nherf3	<i>Nherf3</i> <sup>-/-</sup>	Cimetidine	Decrease	(Shimizu et al., 2011)

Uppercase denotes human genes or gene products and lowercase denotes rodent genes or gene products.



## A. SLC Drug Transporters

### 1. OCTNs

Although the molecular mechanisms of membrane targeting of the OCT and OCTN family are not well characterized, NHERF2, NHERF3, and NHERF4 have been shown to interact with and/or directly regulate OCTN2 function via modulating membrane abundance (Kato et al., 2005; Sugiura et al., 2006; Sugiura et al., 2008; Watanabe et al., 2006). A pull-down study using recombinant C-terminal proteins identified an interaction between apically localized OCTN1 and OCTN2 with both NHERF2 and NHERF3, but not with OCT1 and OCT2, which in contrast, do not harbor PDZ ligands and exclusively localize to basolateral membranes (Kato et al., 2005). Additional studies revealed that the interaction required the presence of the last four amino acids of OCTN1 and OCTN2 (-LTAF and -STAF respectively), both of which are class-I PDZ ligands. The interaction of NHERF3 with the C-terminus of OCTN2 was confirmed in a pull-down study using kidney brush-border membrane vesicles. Subsequent immunohistochemical analysis revealed that NHERF3 and OCTN2 co-localize in mouse kidney brush border membranes. Further, NHERF3 increased OCTN2 mediated uptake of carnitine, a well-established OCTN2 probe substrate. This result could be explained by a 6-fold increase in carnitine transport capacity *in-vitro*. Modulation of OCTN2 function by NHERF3 was not observed upon deletion of the last four amino acids of OCTN2 (-STAF), supporting that these two proteins interact directly via the PDZ ligand of OCTN2 (Kato et al., 2005). An *in-vivo* study performed in *nherf3*<sup>-/-</sup> mice found that absorption of carnitine was decreased compared to wild-type mice (Sugiura et al., 2008). Immunohistochemical analysis revealed that the apical

localization of intestinal *Octn2* was reduced in *nherf3*<sup>-/-</sup> mice compared to wild-type mice (Sugiura et al., 2008). Collectively, these studies suggest a role for NHERF3 in regulating OCTN2 membrane abundance and function in the kidney and intestine. NHERF2 may have a similar role.

Co-expression of OCTN2 and NHERF4 in HEK293 cells also increased the uptake of carnitine (Watanabe et al., 2006). Subsequent kinetic analysis revealed a 2-fold increase in transport capacity but a minimal effect on carnitine specificity for OCTN2, suggesting that the increase in OCTN2 function was not due changes in carnitine affinity, but rather a greater number of OCTNs involved in transport. Further, upon deletion of the last four amino acids in OCTN2 (-STAF), the effect of NHERF4 on OCTN2 was abolished (Watanabe et al., 2006), signifying that the mechanism is dependent on the presence of the PDZ ligand. However, these results were not observed when NHERF4 was co-expressed with OCT3 or OCTN1, despite the fact that both OCT3 and OCTN1 harbor class-I PDZ ligands (-RSHL and -LTAF respectively). These data indicate that NHERF4 might operate by a mechanism that is reliant on structural characteristics other than the existence of a PDZ ligand alone.

Taken together, NHERF proteins may differentially modulate OCTN function secondary to preservation of membrane abundance. These data also point to an uncharacterized NHERF mechanism that operates independent of a PDZ ligand. This hints to involvement of other cytoplasmic adaptor/scaffold proteins or non-canonical binding motifs affecting NHERF binding affinities and thus function. Given that OCTNs have described roles in pharmacokinetics, pharmacodynamics, and pathophysiology, characterizing NHERF mediated OCTN function may lead to a more detailed mechanistic explanation of the pharmacological and pathophysiological role of OCTNs.

## 2. OATs

The OATs play clinically important roles in the disposition of several pharmacologically active compounds as well as many endogenous substances. OAT4 and URAT1 are highly expressed in the kidney and are exclusively found at apical membranes, whereas other renal OAT isoforms (OAT1-3) are found at the basolateral membrane (Burckhardt, 2012). Among the OAT family, OAT4 and URAT1 contain class-I PDZ ligands (-STSL and -STQF respectively) and have been shown to be binding partners with NHERF1 and NHERF3. Evidence suggests that NHERF1 and NHERF3 play a role in regulating OAT4 and URAT1 function (Anzai et al., 2004; Miyazaki et al., 2005; Zhang et al., 2010a; Zhou et al., 2008). Yeast two-hybrid studies and surface plasmon resonance confirmed OAT4 binds both NHERF1 and NHERF3, and that the interaction is mediated by the C-terminal region of OAT4 and the PDZ1 domain of NHERF1, and both PDZ1 and PDZ4 domains in NHERF3 (Miyazaki et al., 2005). The association of OAT4 with NHERF1 and NHERF3 depends on the presence of the C-terminal PDZ ligand of OAT4 (-STSL). Further, association of OAT4 with NHERF1 or NHERF3 enhanced transport of estrone-3-sulfate, an established OAT4 probe substrate, in HEK293 cells. Upon deletion of the OAT4 PDZ ligand, this functional effect was abolished (Miyazaki et al., 2005). These data indicate that NHERF1 and NHERF3 may affect OAT4 function through interactions mediated by specific PDZ domains.

The effect of NHERF proteins on OAT4 function may be tissue specific (Zhou et al., 2008). In a comparison of NHERF mediated OAT4 function in LLC-PK1 versus BeWo cells, a kidney and placental cell line respectively, NHERF1 and NHERF3 only enhanced OAT4 dependent estrone-3-sulfate uptake in LLC-PK1 cells but not BeWo cells. This suggests that the effect of NHERF1 and NHERF3 may be cell or tissue specific. These results further support the hypothesis that NHERF proteins display tissue specific function (Zhou et al., 2008). To evaluate the mechanism of NHERF1 mediated OAT4 regulation, You et al. (2009) showed that protein

kinase-C (PKC), which has previously been shown to inhibit NHERF1 function via phosphorylation, down-regulates OAT4 activity. Further studies in COS-7 cells revealed that NHERF1 attenuates OAT4 internalization (Zhang et al., 2010a). These studies demonstrate that the mechanism of NHERF1 mediated OAT4 regulation may be secondary to preventing OAT4 internalization.

URAT1 has also been studied in the context of NHERF mediated regulation. Similar to OAT4, the C-terminal PDZ ligand of URAT1 (-STQF) is required for interaction with NHERF3 and for NHERF3 dependent functional regulation (Anzai et al., 2004). In HEK293 cells, urate transport was increased by NHERF3, an effect that was abolished upon deletion of the URAT1 C-terminal PDZ ligand. Kinetic analysis revealed that this effect was due to an increase in the capacity of urate transport rather than a change in urate specificity for URAT1. Subsequent co-immunoprecipitation studies showed that URAT1 co-localizes with NHERF3 at the apical brush border membrane in renal proximal tubule cells and the presence of the URAT1 PDZ ligand is required for this interaction (Anzai et al., 2004).

Together, these studies highlight an important role for NHERF1 and NHERF3 in regulating OAT4 and URAT1 function. Although both transporters have been proposed to play important pharmacological roles, they also have been shown to mediate the renal secretion and reabsorption of uric acid (Graessler et al., 2006; Sakiyama et al., 2014). This implies that in addition to potential effects on renal drug secretion, NHERF proteins may also influence uric acid homeostasis and may have an underlying role in the pathophysiology of gout.

### 3. OATPs

Five members of the OATP family harbor a class-I PDZ ligand at their C-terminus (Table 2). Evolutionarily, the OATPs are poorly conserved and orthologs in rodents may not exist in humans making findings from studies performed in animal models difficult to translate clinically. Although multiple studies indicate that NHERF proteins play a role in regulating rodent Oatps (Choi et al., 2011; Sugiura et al., 2010; Wang et al., 2005; Wang et al., 2014), there is little information regarding how NHERF proteins affect human OATP isoforms.

OATP1A2 was the first identified human OATP isoform and has since been well studied and found to play an important role in the cellular uptake of a variety of drugs and endogenous substances. Recently, NHERF1 and NHERF3 were found to regulate OATP1A2 function by modulating protein internalization and enhancing membrane stability. The influence of NHERF1 and NHERF3 on OATP1A2 function was assessed using HEK293 cells that co-expressed NHERF1 or NHERF3 (Zheng et al., 2014). Similar to studies in OATs, estrone-3-sulfate uptake was utilized as a surrogate for OATP1A2 function. Both NHERF1 and NHERF3 were found to significantly enhance E<sub>1</sub>S uptake. Overexpression of NHERF1 or NHERF3 leads to an increase in OATP1A2 membrane abundance and was proposed to be the underlying basis for the observed increase in OATP1A2 function. Subsequent studies revealed that the mechanism for the apparent increase in OATP1A2 membrane abundance was due to a decrease in its internalization via a clathrin-dependent, but caveolin-independent, manner (Zheng et al., 2014). NHERF proteins may have similar effects on the other human OATPs that contain C-terminal PDZ ligands (Table 2). Particular attention should be given to OATP2B1 due to its established functional role in the intestine as an uptake transporter for several drugs including fexofenadine and statins. OATP2B1 also uniquely displays dual-membrane specificity, apical in enterocytes and basolateral in hepatocytes (Figure. 1). This may indicate that if NHERF proteins are

involved in regulating OATP2B1, they may do so differently in the intestine than in the liver. Investigating the mechanism that determines intestinal versus hepatic OATP2B1 membrane targeting may provide clues on how NHERF proteins, or other PDZ proteins, function in different tissues.

#### 4. PEPTs.

PEPT1 and PEPT2 are restricted to apical membranes in the intestine and kidney. Recent evidence shows that NHERF2 and NHERF3 interact with PEPT2 and enhance transport activity by preserving PEPT2 membrane abundance (Boehmer et al., 2008; Kato et al., 2004; Noshiro et al., 2006; Sugiura et al., 2006). A preliminary screening of several drug transporters and PDZ proteins identified a potent interaction between PEPT2 and NHERF3 (Kato et al., 2004). Studies using yeast two-hybrid assays and surface plasmon resonance confirmed the interaction between PEPT2 and NHERF3 is mediated by the PDZ2 and PDZ3 domains (Noshiro et al., 2006). The influence of NHERF3 on PEPT2 transport was subsequently assessed in HEK293 cells using Gly-Sar uptake as a surrogate of PEPT2 function (Noshiro et al., 2006; Sugiura et al., 2006). Co-expression of NHERF3 in HEK293 cells augmented PEPT2 function and was associated with an increase in transport capacity. Co-expression with NHERF3 increased PEPT2 membrane abundance and was proposed to account for the increase in function. Tsuji et al. (2006) later evaluated PEPT2 by assessing Gly-Sar uptake in HEK293 cells stably expressing NHERF3 constructs with a mutation in the PDZ2 domain (Sugiura et al., 2006). Although HEK293 cells overexpressing the NHERF3 mutant displayed a decrease in Gly-Sar uptake compared to those overexpressed with wild-type NHERF3, the results were not significant (Sugiura et al., 2006). Identifying the effects of genetic polymorphisms in NHERF

proteins on drug transport may help clarify which drug transporters are subject to NHERF regulation. Revealing which PDZ domains are important for drug transporter regulation also may provide detail on the structural requirements of NHERF function.

Despite similarities in structure and domain organization between NHERF1 and NHERF2, expression studies performed in *Xenopus* oocytes have shown that NHERF2, but not NHERF1, enhance PEPT2 membrane abundance and function. This is another example of NHERF target-proteins displaying differential binding affinities for NHERF isoforms. The effect of NHERF2 on PEPT2 was not observed upon deletion of the C-terminal PDZ ligand of PEPT2 (-KTKL), suggesting that the PEPT2 PDZ ligand is a requirement for interaction with NHERF2 (Boehmer et al., 2008). Why NHERF1 does not produce similar effects, despite its similarity with NHERF2, is uncertain.

Collectively, these studies indicate that NHERF proteins modulate PEPT2 function and therefore may play important physiological and pharmacological roles in oligopeptide and peptide-like drug transport in the kidney. However, several questions remain regarding the regulation of PEPT membrane targeting. For instance, it is unclear why PEPT2 preferentially binds NHERF2 and NHERF3, but not NHERF1, especially considering NHERF1 and NHERF3 are highly expressed in the kidney, where PEPT2 is also prominently expressed. A closer inspection of PEPT2 regulation by NHERF proteins in the kidney may reveal cooperative or opposing roles for NHERF1 and NHERF3. This uncertainty is an opportunity to flesh out details regarding regulation mediated by multiple NHERF isoforms. Alternatively, NHERFs might regulate PEPT transport indirectly. NHERF3 may regulate PEPT1 as described (Kato et al., 2006). PDZ proteins, such as NHERFs, may influence drug transport indirectly by regulating proteins that are responsible for producing electrochemical gradients necessary for dissipative transport. For example, PEPT1 utilizes an inwardly directed  $H^+$  gradient that is produced by the  $Na^+/H^+$  exchanger (NHE) to provide the driving force for substrate uptake. NHERFs may indirectly regulate PEPT1 by directly regulating NHE or recruiting NHE in close proximity to

PEPT1 to ensure an adequate H<sup>+</sup> gradient is achieved. Discovering the nature of the functional coupling of PEPT1 and NHE by NHERFs, as well as differences in NHERF-dependent PEPT membrane targeting, may broaden our understanding of NHERF action and drug transport. These studies will aid in understanding how PEPTs are organized at cell membranes, and lead to new discoveries regarding how PDZ proteins, including NHERF proteins, interact with each other to perform non-redundant physiological functions in different tissues.

## B. ABC Drug Transporters

### 1. MRPs

NHERF1 and NHERF3 have been reported to regulate the membrane abundance, localization, and function of MRP2 (-STKF) and MRP4 (-ETAL), both of which contain a class I C-terminal PDZ ligand. The initial discovery that NHERF proteins interact with MRPs resulted from studies that focused on elucidating the cellular mechanisms responsible for multidrug resistance in cancer after it was found that NHERF3 is up regulated in selected tumors of epithelial origin (Kocher et al., 1999; Kocher et al., 1998). This prompted an investigation of potential NHERF3 binding partners. Expression studies and yeast two-hybrid assays performed in human carcinomas confirmed an increase in NHERF3 mRNA and revealed that NHERF3 interacts with the C-terminal portion of MRP2 (Kocher et al., 1999). Later, it was revealed that MRP2 interacts with all four NHERF isoforms (NHERF1-4) and that phosphorylation of Ser1542 in the MRP2 PDZ binding motif significantly enhanced binding to NHERF1 and NHERF4; the functional



consequence was not assessed (Hegedus et al., 2003). Because of these findings, others have investigated the role of intracellular kinases, such as PKC, and membrane localization of MRP2. For example, specific PKC-isoforms, such as conventional PKC $\alpha$  (cPKC $\alpha$ ), novel PKC $\delta$  (nPKC $\delta$ ), nPKC $\epsilon$ , and atypical PKC $\zeta$  (aPKC $\zeta$ ), appear to be involved in regulating hepatic MRP2 membrane recycling at the apical/canalicular membrane (Beuers et al., 2003; Ito et al., 2005; Park et al., 2012; Schonhoff et al., 2008; Stross et al., 2009). It is still unclear if these PKC-mediated phosphorylation events result from direct phosphorylation of MRP2, NHERF proteins, or members of the ERM family, such as radixin, known to interact with and influence MRP2 function (Suda et al., 2014; Suda et al., 2011). Investigating phosphorylation events important for drug transporter regulation represents an emerging line of research that intersects with the physiological and pharmacological role of PDZ proteins.

To describe further the role of NHERF1 dependent MRP2 regulation in the liver, *nherf1*<sup>-/-</sup> mice have been employed. In livers obtained from *nherf1*<sup>-/-</sup> mice, Mrp2 mRNA was unchanged but protein levels were significantly decreased compared to wild-type mice, suggesting post-transcriptional changes in Mrp2 (Li et al., 2010). Immunofluorescent staining of Mrp2 in hepatocytes from *nherf1*<sup>-/-</sup> mice showed that although a pool of Mrp2 remained at the apical/canalicular membrane, the signal was noticeably weaker. Bile flow in *nherf1*<sup>-/-</sup> mice was reduced by 70%, compared to wild-type mice, with a 50% and 60% reduction in glutathione and glutathione-methylfluorescein biliary excretion in isolated hepatocytes. Bile acid and bilirubin excretion, surrogates of hepatic MRP2 function, were unchanged (Li et al., 2010). Studies performed in *nherf1*<sup>-/-</sup> provide strong evidence of the importance of NHERF1 in targeting and stabilizing hepatic MRP2 to the apical/canalicular membrane. In a more recent study, fluorescent-tagged mutant constructs were utilized in WIF-B cells, a polarized hepatocyte cell-line, to study localization and function of NHERF1 (Karvar et al., 2014). Fluorescence microscopy was used to visualize the cellular distribution of NHERF1, MRP2, and radixin. A point mutation in the radixin binding site of NHERF1 (F335R) as well as deletion mutations

(PDZ1 and PDZ2) were used to assess NHERF1 membrane association and a 5-chloromethylfluorescein diacetate (CMFDA) assay was used to characterize MRP2 function. It was later confirmed that only overexpression with the radixin binding site mutant (F335R) and the PDZ1 deletion mutant resulted in decreased NHERF1 membrane association and MRP2 function (Karvar et al., 2014). These results support that the functional importance of NHERF1 depends on its interaction with MRP2 and the PDZ1 domain and demonstrate that NHERF1 is important for the distributional dynamics of MRP2 in the liver. Although NHERF3 was the initial NHERF isoform identified to interact with MRP2 (Kocher et al., 1999), subsequent studies performed in kidney proximal tubule cells of *nherf3*<sup>-/-</sup> mice revealed no change in expression or cellular distribution of Mrp2 (Kocher et al., 2003). This indicates that NHERF3 is not involved in MRP2 membrane targeting. Alternatively, functional compensation by other PDZ proteins or cytoplasmic adapters may preserve MRP2 membrane abundance in the absence of NHERF3 and thus be confounding these results.

The anti-viral drug adefovir is an established MRP4 probe substrate and is often used as a surrogate of MRP4 function. As noted earlier, MRP4 is critical for the renal elimination of adefovir (Imaoka et al., 2007). NHERF1 and NHERF3 also regulate MRP4 membrane localization and function; however, they appear to have opposing roles, as down regulation of NHERF1 increases MRP4 function, whereas NHERF3 stabilizes MRP4 at apical membranes and increases function (Hoque and Cole, 2008; Hoque et al., 2009; Park et al., 2014). Knock down of NHERF1 by small interfering RNA (siRNA) in HeLa cells increases MRP4 membrane abundance and reduces cellular accumulation of the MRP4 probe substrates, 6-mercaptopurine and 9-[2-(phosphonylmethoxy) ethyl]-adenine (Hoque and Cole, 2008). The authors attributed these results to a decrease in MRP4 internalization mediated by NHERF1. It was later demonstrated that MRP4 localizes to either apical or basolateral membranes depending on the cell type and the degree of NHERF1 expression (Hoque et al., 2009). This study found that MRP4 localizes to basolateral membranes in MDCKI cells, where NHERF1 expression is low,

whereas MRP4 localizes to apical membranes in LLC-PK1 cells, which express a higher amount of NHERF1 (Hoque et al., 2009). Although these studies provide proof-of-principle, *in-vitro* cell systems are limited and may not represent the endogenous role(s) of NHERF proteins and drug transporter regulation. Further, PDZ regulation of drug transporters are not likely to depend on a single PDZ isoform but rather multiple isoforms, as well as on members of the ERM family. For these reasons, extrapolating these data to *in vivo* systems will advance our understanding of PDZ regulatory processes.

In contrast to NHERF1, NHERF3 is critical for MRP4 dependent efflux in the kidney. The role of NHERF3 on MRP4 membrane targeting and function was assessed in an elegantly conducted study using *nherf3*<sup>-/-</sup> mice and HEK293 cells, (Park et al., 2014). MRP4 protein expression, membrane abundance, and function decreased in *nherf3*<sup>-/-</sup> mice compared to wild-type mice. A pharmacokinetic study evaluated the role of NHERF3 on MRP4 dependent renal drug elimination (Park et al., 2014). Mice were administered an intravenous injection of [<sup>3</sup>H]-adefovir, an established prototype MRP4 probe substrate that undergoes negligible metabolism and is predominately eliminated via the kidney. Plasma clearance of [<sup>3</sup>H]-adefovir was significantly decreased in *nherf3*<sup>-/-</sup> mice compared to wild type mice. This result was accompanied by a significant increase in the [<sup>3</sup>H]-adefovir concentration area under the curve (AUC<sub>0-inf</sub>), which is reflective of the systemic exposure of [<sup>3</sup>H]-adefovir. Further, the concentration of [<sup>3</sup>H]-adefovir in kidneys was significantly higher in *nherf3*<sup>-/-</sup> mice compared to wild-type mice suggesting impaired MRP4 mediated efflux from renal proximal tubules (Park et al., 2014). Collectively, these pharmacokinetic changes suggest that NHERF3 plays a crucial role in MRP4 dependent renal drug elimination as a regulator of MRP4 apical membrane insertion in proximal tubule epithelial cells, and thus an indirect contributor to drug clearance via the kidney. Immunoprecipitation assays using transfected HEK293 cells revealed that the interaction between NHERF3 and MRP4 is mediated by the C-terminal portion of MRP4 (-ETAL) and the PDZ1 domain of NHERF3 (Park et al., 2014). When co-expressed in HEK293 cells,

surface biotinylation and internalization assays revealed that NHERF3 increases MRP4 membrane stability and reduces MRP4 internalization (Park et al., 2014). Pharmacokinetic studies that utilize *nherf1*<sup>-/-</sup> and *nherf3*<sup>-/-</sup> mice represent the next stage in translating the pharmacological significance of NHERF proteins and drug transport.

Several questions remain regarding how NHERF1 and NHERF3 affect MRP4 function and why they seemingly exhibit opposite actions. It is unclear why MRP4 localizes to basolateral membranes in hepatocytes rather than the apical membrane, as it does in renal proximal tubules. Although NHERF proteins are likely involved in MRP4 membrane organization and function, members of the ERM family may also direct MRP4 targeting, as they do with MRP2. Investigating the interaction between NHERF proteins, MRP2, MRP4, and members of the ERM family may provide additional details regarding tissue specific mechanisms.

## V. PDZ PROTEINS AND DRUG ACTION IN DISEASE STATES

### A. Cancer.

The ABC superfamily of drug transporters is well known in oncology for their role in conferring multidrug resistance (MDR) (Gottesman et al., 2002). ABC transporters actively efflux a broad array of chemotherapeutic agents such as taxanes, camptothecins, and platinum agents, thus contributing to decreased intracellular drug accumulation in tumor cells and ultimately failure of chemotherapy (Gottesman et al., 2002). Pgp (*MDR1*), MRP1 (*ABCC1*), and BCRP (*ABCG2*), the ABC superfamily, play important roles in MDR (Doyle et al., 1998; Kunicka and Soucek, 2014; Wu et al., 2014). Overexpression of these transporters is well documented in many types of cancer, and considered to be the underlying mechanism for chemoresistance attributable to ABC drug efflux in tumors (Szakacs et al., 2006; Teodori et al., 2006; Wu et al., 2014).

Substrate-induced induction is one mechanism involved in the upregulation of ABC drug transporters, where chemotherapeutic drugs enhance chemoresistance through nuclear receptors involved in the transcriptional expression of efflux drug transporters (Chen, 2010; Herraes et al., 2012; Manceau et al., 2012; Oda et al., 2013). For example, the pregnane X receptor (PXR) is a nuclear receptor that binds promiscuously with many structurally diverse ligands, including anti-cancer drugs such as paclitaxel (Harmsen et al., 2009; Harmsen et al., 2010). The expression of PXR has been characterized in many cancers where it is thought to be involved in resistance to chemotherapy. Upon ligand activation, PXR forms a heterodimer with other nuclear receptors, such as the retinoid X receptor, and induces the expression of target

genes involved in drug resistance. It has been clearly demonstrated that PXR directly regulates Pgp expression (Harmsen et al., 2010). To address the problem of MDR in cancer, novel host- and tumor-mediated pathways continue to be explored in an effort to identify potential new therapeutic targets.

The clinical contribution to MDR from other drug transporters remains uncertain. However, in an evaluation of ABC drug transporters in a panel of tumor cell lines from the US National Cancer Institute, over half of the members that comprise the ABC drug transporter family have been implicated as playing a role in conferring MDR, suggesting multiple drug transporters are likely involved (Szakacs et al., 2004). As a means to combat MDR in cancer patients, targeting ABC drug transporters through pharmacological inhibition has been attempted for several years but with poor results (Karthikeyan and Hoti, 2015; Turk and Szakacs, 2009). Due to their clinical contribution to MDR, studies aimed at understanding the regulatory mechanisms of transporters focus heavily on changes in gene and protein expression. Studies that investigate post-translational modification of drug transporters in cancer remain an area of research that is underrepresented. Future studies aimed at expanding our knowledge of drug transporter regulation in cancer outside the realm of transcriptional regulation are warranted.

The NHERF proteins, particularly NHERF1, have been implicated in different types of cancers. Although its role as a tumor suppressor and/or oncogenic protein remains controversial, overexpression of NHERF1 in several cancers, such as breast cancer, schwannoma, and hepatocellular carcinoma has been described (Cardone et al., 2007; Fraenzer et al., 2003; Shibata et al., 2003; Stemmer-Rachamimov et al., 2001). In addition, tumor stage, metastatic progression, poor prognosis, and estrogen receptor status are significantly associated with NHERF1 overexpression (Cardone et al., 2007; Song et al., 2007). Further, NHERF1 upregulation in conditions of serum deprivation and hypoxia suggest that NHERF1 function may increase in a tumor microenvironment (Cardone et al., 2007). Although

NHERF1 expression increases in human cancer, its cellular distribution appears to shift from the plasma membrane to nuclear and cytoplasmic regions (Cardone et al., 2007; Fraenzer et al., 2003; Stemmer-Rachamimov et al., 2001). The functional consequences of the observed shift in the cellular distribution of NHERF1 in cancer continue to be investigated.

The mechanisms of MDR in cancer are likely multifactorial. In addition to alterations in transcriptional expression, post-translational modification of drug transporters may also be affected. The reported changes in NHERF1 expression in cancer combined with the newly described role of NHERF1 in drug transporter function, underscores NHERF1 as an attractive and novel MDR target that is worthy of investigation. A critical evaluation of the role of NHERF1, as well as other NHERF isoforms, and drug transporter function in cancer may offer mechanistic insight into chemoresistance. Overexpression of select drug transporters, such as those believed to play major roles in cancer (Pgp, MRP1, and BCRP), may represent only one of several mechanisms that contribute to MDR. Although not considered to confer significantly to MDR, ABC drug transporters that contain Class-I PDZ ligands, such as MRP2 and MRP4, are implicated in chemoresistance (Kool et al., 1997; Zhang et al., 2010b). Therefore, the observed reduction in drug action may be partially attributable to altered membrane targeting and/or abundance of drug transporters involved in efflux of chemotherapeutic agents. Faulty membrane targeting may not result in observable changes in mRNA or protein expression of drug transporters in cancer and therefore those transporters subject to NHERF regulation may be overlooked despite their potential involvement in conferring resistance. Turning attention to NHERF1 as a mediator of drug transporter regulation in cancer may lead to a more complete understanding of MDR while also highlighting NHERF1 as a potential new target.

## B. Chronic Kidney Disease.

A well-known consequence of chronic kidney disease (CKD) is reduced renal drug clearance. Dosing guidelines have been developed for drugs that undergo renal elimination, where doses are adjusted according to some measure of kidney function (i.e., creatinine clearance or glomerular filtration rate) based on the premise that renal clearance declines in proportion to kidney function (Nielsen et al., 2014). Despite the development of renal dosing guidelines, patients with CKD experience high rates of adverse drug events (Zaidenstein et al., 2002). However, both renal and extra-renal drug clearance mediated by key drug transporters is altered in patients with CKD (Nolin et al., 2009). This undoubtedly contributes to the high variability in drug response and the increased frequency of adverse drug events observed in CKD patients (Bates et al., 1999; Matzke and Frye, 1997; Nolin and Unruh, 2010; Zaidenstein et al., 2002). Changes in drug transporter gene and protein expression in both renal and extra-renal tissues do not consistently correlate with the expected changes in transporter function in the setting of CKD. For instance, intestinal Pgp function is reduced *ex vivo* in the intestine of 5/6<sup>th</sup> nephrectomized rats, an experimental model of CKD (Veau et al., 2001). Interestingly, no change in Pgp mRNA or protein expression was observed, indicating that changes in transcriptional expression could not explain the reduction in function. Later, others confirmed and extended these results to other transporters. In 5/6<sup>th</sup> nephrectomized rats, a significant decrease in Pgp, Mrp2, and Mrp3 protein expression was observed with no change in mRNA (Naud et al., 2007). These discrepancies are not limited to the intestine. Additional studies utilizing the same experimental animal model of CKD show that several hepatic and renal drug transporters, such as members from the Oatp, Oat, Oct, Pgp, and Mrp families, also display changes in expression that do not correlate with function (Ji et al., 2002; Laouari et al., 2001; Naud et al., 2008; Tsujimoto et al., 2008). However, data across studies yield highly variable



and inconsistent results, which makes interpretation difficult. Further, despite these important findings, there is currently no clear mechanism as to the cause. These discrepancies and lack of a definitive mechanism for changes in transporter function in CKD limits the extrapolation of these data into the clinical setting.

To date, studies aimed at evaluating changes in gene and protein expression alone in CKD have not reconciled this disparity. Many studies attribute uremic toxins, metabolic byproducts that accumulate in uremia, as a major cause of reduced drug transporter function in CKD (Barreto et al., 2014; Masereeuw et al., 2014). Uremic toxins are believed to competitively inhibit drug transport and to down regulate transporter expression (Reyes and Benet, 2011; Sato et al., 2014). However, uremic toxins do not begin to accumulate until end-stage renal disease (ESRD) and therefore may only explain changes in drug transporter function in patients with ESRD. Moreover, hundreds, if not thousands of uremic toxins may exist (Masereeuw et al., 2014; Massy, 2014; Vanholder et al., 2003). Determining which toxin(s) have the greatest impact on transporter function has also proved difficult. The mechanisms responsible for reduced drug transporter function may be more complex and begin in earlier stages of CKD, well before reduced kidney function limits uremic toxin elimination. Novel mechanistic hypotheses that address altered drug transporter function in CKD are needed to advance our understanding of this field and ultimately develop dosing strategies to prevent adverse events in this patient population.

CKD represents a disease state for which exploration of NHERF dependent drug transporter regulation may provide novel insights into altered transporter function (Figure. 5). How drug transporter function changes in CKD from the standpoint of NHERF dependent transport has yet to be investigated. This is likely because only recently has research in drug transporter membrane targeting gained interest. The NHERF family is abundantly expressed in the intestine, liver, and kidney, tissues that also highly express transporters. To date, there are no data on changes in NHERF function in CKD. Consideration should be given to understanding

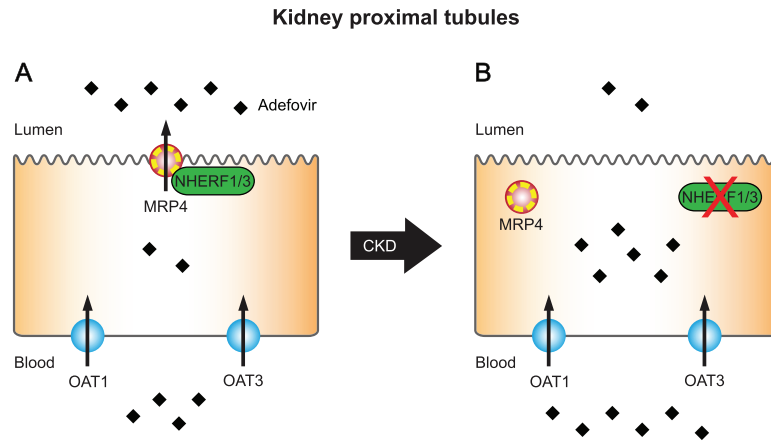


Figure 5

how NHERF function may change over the course of CKD and the implications on drug transport.

## VI. CONCLUSIONS AND FUTURE DIRECTIONS

The functional regulation of drug transporters by the NHERF family continues to capture the attention of those who investigate the underlying mechanisms that govern pharmacokinetics and pharmacodynamics. This relatively novel understanding of NHERF is still in its infancy, as several questions remain surrounding the NHERF family and regulation of drug transport. Future studies in this area should focus on addressing 1) the NHERF isoforms affecting drug transport and which transporters are the most susceptible to regulation by NHERF; 2) the different mechanisms of NHERF-directed membrane targeting depending on tissue type; 3) the functional importance of NHERF dimerization and cooperativity as it relates to drug transport 4) the role of the ERM family in NHERF/drug transporter interactions; and 5) the role of NHERF proteins in MDR in cancer and altered drug transport function in CKD. Additional pharmacokinetic studies using specific phenotypic probe substrates of drug transport performed in *nherf1*<sup>-/-</sup> and *nherf3*<sup>-/-</sup> mice would provide an excellent model to begin addressing these gaps in our understanding. However, data performed in mouse models eventually must be translated to humans. The inability to assess NHERF non-invasively in humans represents an important limitation to this work. However, mutations in the human NHERF1 (*SLC9A3R1*) gene do exist (Karim et al., 2008). Clinical pharmacokinetic studies performed in patients who carry polymorphisms in the NHERF1 (*SLC9A3R1*) gene, may provide a means to address this limitation.

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